Cytochrome c_4 - Characterisation, Location and Effect of Growth Conditions

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The work described in this thesis is my own, unless otherwise indicated. Some of the results are published in Leitch, F.A., Brown, K.R. & Pettigrew, G.W. (1985) Biochim. Biophys. Acta 808, 213-218 and Goodhew, C.F., Brown, K.R. & Pettigrew, G.W. (1986) Biochim. Biophys. Acta 852, 288-296, which are bound in Appendix I, and Pettigrew, G.W. & Brown, K.R. (1988) Biochem. J. 252, 427-435

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ABSTRACT

The work described in this thesis deals with the properties, cellular location, effect of growth conditions and the redox properties of cytochrome c_4 from the bacterium Pseudomonas stutzeri 224.

Spectrally cytochrome c_4 is characterised by an α-peak maximum at 550nm and an α/β ratio of 1.22-1.23. The mobility on SDS gels is dependent on the redox state of the haem iron and whether the haem is present or not.

The cellular location of cytochrome c_4 was identified as being mainly membrane bound by purification of cytochrome c_4 from both soluble and membrane fractions. Analysis of cytoplasmic, periplasmic and membrane fractions by haem stained gels confirmed that cytochrome c_4 is mainly membrane bound and in addition demonstrated that soluble cytochrome c_4 is located in the periplasm. Proteolysis experiments on right-side-out vesicles demonstrated that cytochrome c_4 is associated with the periplasmic face of the membrane. Cytochromes c_4 from soluble and membrane fractions of aerobic and nitrate grown cells were shown to be identical with respect to amino acid composition, spectra, ELISA and redox titration.

The levels of cytochrome c_4 were shown to be virtually identical in both aerobic and nitrate grown Pseudomonas stutzeri , however, the distribution of cytochrome c_4 between membrane and soluble fractions was

affected by growth conditions with more membrane bound cytochrome c_4 found in aerobically grown cells.

Redox titration of cytochrome c_4 yielded sigmoidal Nernst plots which may be analysed in terms of two components of +300 and +190mV. Two models are proposed to explain the two redox potentials; (1) the haems are intrinsically different or (2) the haems have identical potentials in the oxidised form but addition of one electron makes the addition of a second much more difficult (negative cooperativity). Proteolytic cleavage of the cytochrome with chymotrypsin yielded two fragments, one with a molecular weight of approx. 10000, a 695nm band and a midpoint potential of +110mV. The second fragment was damaged, as determined by the loss of the 695nm band, had a molecular weight of approx. 6000 and a midpoint potential of -190mV. Without two undamaged fragments it was not possible to distinguish between the two proposed models of reduction.

A partial characterisation of a c-type cytochrome of approx. 30000 molecular weight, which is greatly induced under nitrate growth, is also reported.

CONTENTS

CHAPTER I : INTRODUCTION

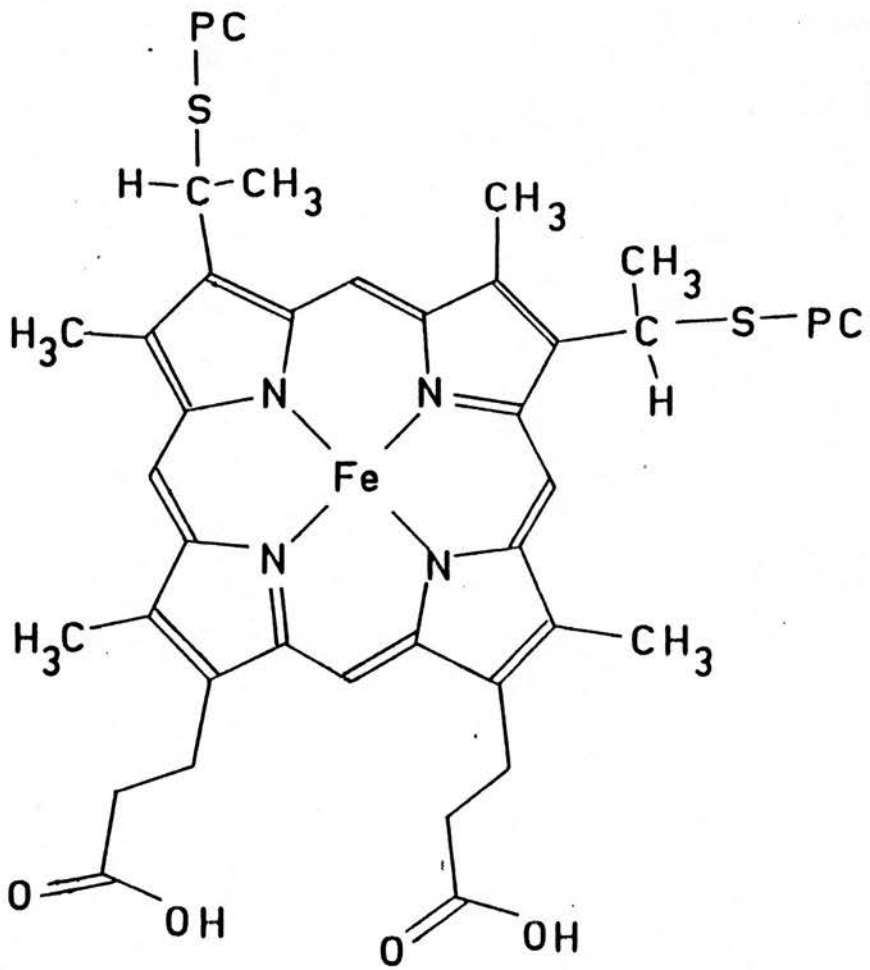
Section I - c-Type Cytochromes

The c-type cytochromes are a group of electron transfer proteins having one or more protohaem IX (see Figure 1) prosthetic groups which are covalently attached to the polypeptide chain via thioether linkages. These linkages result from condensation of the two haem vinyl groups of the haem moiety and the two cysteines of the polypeptide chain. However, examples are known where the protein moiety has only one cysteine residue and thus the haem is bound by only one thioether linkage. An example of a c-type cytochrome with only one thioether linkage is a c-type cytochrome from Crithidia oncopelti (Pettigrew 1972).

The haem iron has five or six co-ordinating ligands, the number of ligands determining the spin state. Low spin cytochromes have six co-ordinating ligands while high spin cytochromes generally have five (high spin may have six coordinating ligands if the 6th is weak field) (Pettigrew and Moore, 1987). It is possible in the case of multihaem c-type cytochromes for all the haems to be in the same spin state (eg the four haems of cytochrome c_3 are low spin (Matthews et al, (1972)) or the haems can be in different spin states (eg three of the haems in tetrahaem cytochrome c-554 from Nitrosomonas europaea are low spin while the remaining haem is high spin (Andersson et al, (1986)).

Figure 1 : Haem c

Showing sites of covalent attachment to the polypeptide chain (PC).


PC
S
H
C
CH3
CH3
CH3
C
S
PC
H
H3C
N
N
Fe
N
N
H3C
CH3
O
OH
O
OH

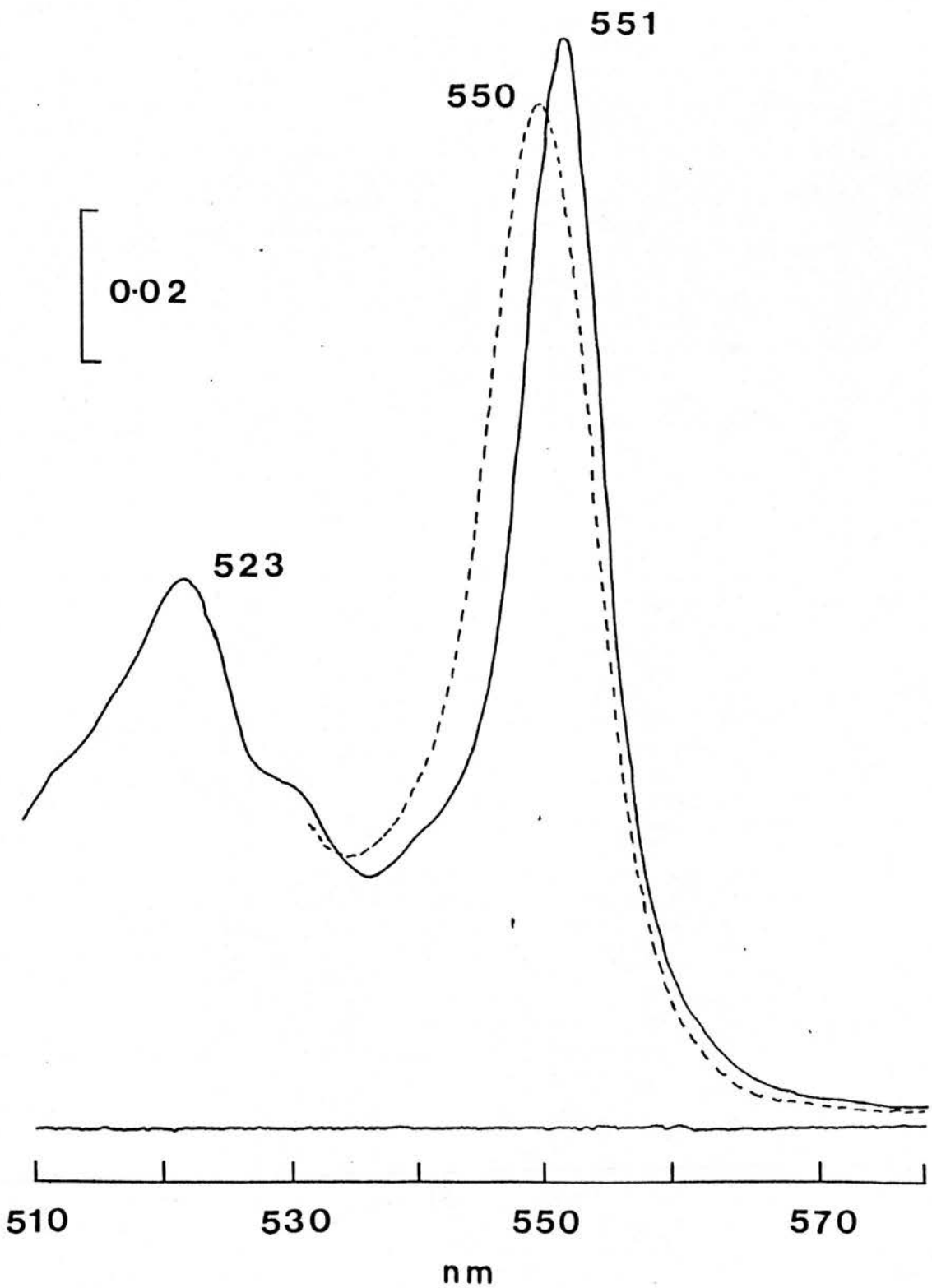
The four pyrrole nitrogens of porphyrin provide the planar co-ordinating ligands. The other ligand(s) is provided by the protein moiety. In all c-type cytochromes the 5th axial ligand is histidine. Low spin c-type cytochromes generally have methionine as the 6th co-ordinating ligand. This histidine-methionine ligation can be identified spectrophotometrically by the presence of an absorption band near 695nm when the cytochrome is in the oxidised form. Cytochrome c_3 is an example of a low spin cytochrome in which histidine forms the 6th co-ordinating ligand (Matthews et al, 1972). Cytochromes with histidine-histidine ligation do not exhibit a 695nm absorption band.

High spin cytochromes generally have no 6th co-ordinating ligand . Spectrally high spin c-type cytochromes can be recognised by a broad absorption band in the region of 630nm when the cytochrome is in its oxidised form.

The haem prosthetic group confers a distinctive absorption spectrum on the c-type cytochrome in the range 500-580nm. Figure 2 shows the absorption spectrum of reduced, native cytochrome c-551 (solid line) from Pseudomonas stutzeri 224. The c-type cytochromes generally have α-peak maxima in the region of 550-555nm and β-peak maxima around 522nm. The broken line represents the pyridine ferrohaemochrome. To obtain such a spectrum, reduced cytochrome is denatured in a solution of sodium hydroxide, exposing the haem group. Pyridine then co-ordinates with the haem iron producing a spectrum

Figure 2 : Spectra of Pseudomonas stutzeri 224 Cytochrome c-551

Solid line shows the dithionite reduced spectrum. Broken line shows the pyridine ferrohaemochrome.


551
550
0·02
523
510
530
550
570
nm

with an α-peak maximum at 550nm, this being indicative of a c-type cytochrome.

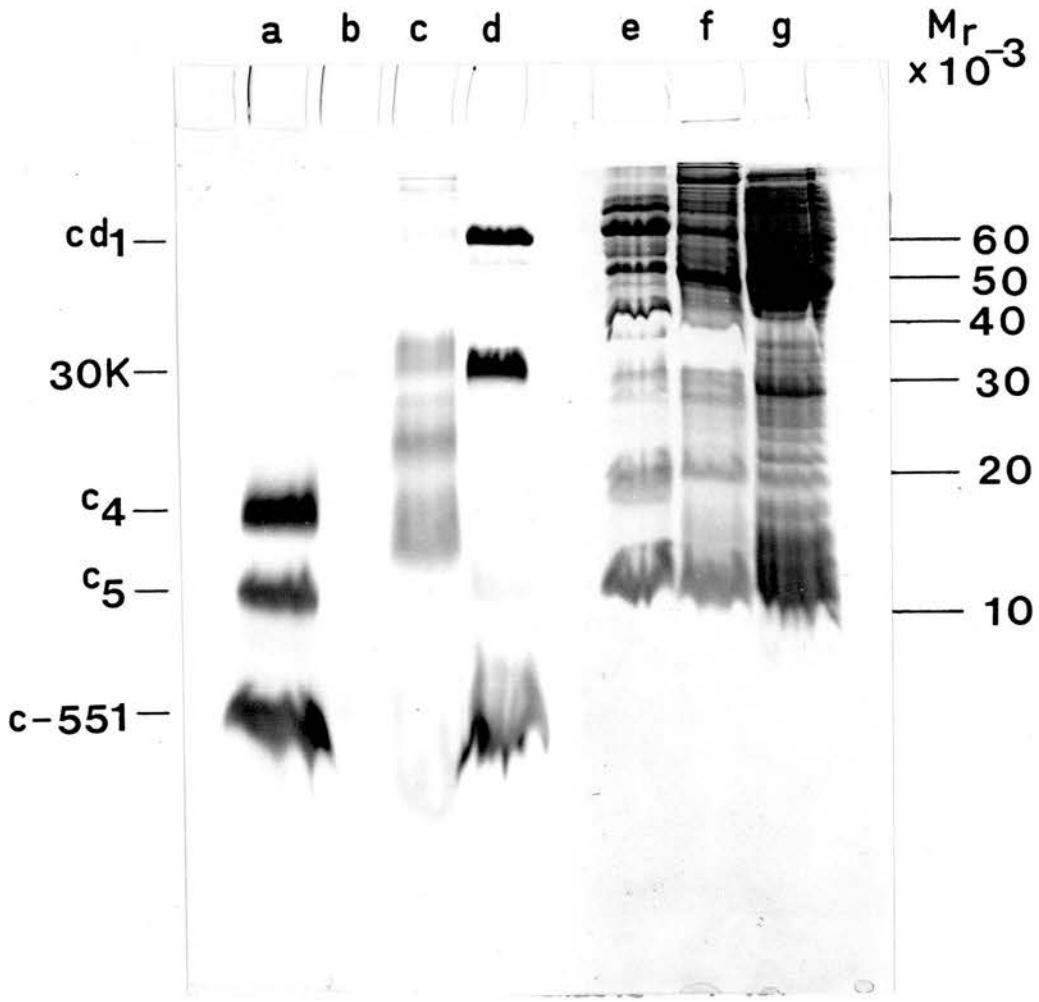
The exposed haem also possesses peroxidase activity - ie reduces hydrogen peroxide to water. This haem peroxidase activity can be exploited to detect proteins which retain haem in SDS. After polyacrylamide gel electrophoresis c-type cytochromes are detected by their haem peroxidase activity using tetramethylbenzidine (TMBZ) as electron donor to the reaction. Oxidation of the TMBZ produces a blue precipitate at the position of c-type cytochromes within the gel (Thomas et al, 1976). This technique has proved valuable in identifying c-type cytochromes in a mixture of proteins. Figure 3 demonstrates the power of staining gels for haem peroxidase activity. The right hand lanes were samples taken from cytoplasm, periplasm and membranes stained for protein with Coomassie blue. These show large numbers of protein bands. However, if the samples were stained for haem, very few bands are seen.

Many of the c-type cytochromes are soluble and available in large quantities making them ideal candidates for purification and characterisation. As a consequence of their small size and ease of purification, more than 140 cytochromes, from both eukaryotic and prokaryotic origin, have had their amino acid sequences determined. From these sequence data Ambler proposed a classification scheme of four classes, termed I-IV (Ambler, 1980).

Class I is the largest class, which contains the cytochromes which are homologous to mitochondrial

Figure 3 : SDS-PAGE Analysis of Cellular Extracts from Pseudomonas stutzeri 224

The gel shows the power of utilising the peroxidase activity of haem to locate c-type cytochromes in complex mixtures of proteins. Lanes (a)-(d) were stained for haem and (e)-(g) stained for protein. Lane (a), 0.05 nmole of purified cytochromes c_4, c_5, and c-551; lanes (b) and (g): cytoplasmic fractions; lanes (c) and (f): membrane fractions; lanes (d) and (e): periplasmic fractions. All fractions were isolated from denitrifying Pseudomonas stutzeri 224. The M_r scale was constructed from the relative mobilities of a set of molecular weight standards.


a
b
c
d
e
f
g
Mr
x10-3
cd1
30K
c4
c5
c-551
60
50
40
30
20
10

cytochrome c. The characteristics of the class include: (a) they are low spin with histidine and methionine as axial haem ligands (b) they generally have higher redox midpoint potentials (+150 to +400 mV) than the other classes (c) the polypeptide chain ranges from 80-134 amino acid residues in length (d) they have 1 haem covalently bound near the N-terminus (cytochrome c_4, with two haems and approximately 180 amino acid residues, is a member of class I, but this will be discussed in Chapter I Section III) (e) the members of Class I are structurally very similar despite the variation in their polypeptide chain lengths resulting from various insertions and deletions, and (f) in general they act as diffusible electron transport proteins, acting between a reductase, which may be membrane bound (eg cytochrome c reductase, the bc_1 complex) or soluble (eg methanol dehydrogenase) and a terminal acceptor, which again may either be membrane bound (eg cytochrome c oxidase, cytochrome aa_3) or soluble (eg cytochrome cd_1, nitrite reductase). A possible exception to this may be cytochrome c_4, which is membrane bound.

Specific examples of Class I cytochromes include mitochondrial cytochrome c, cytochrome c_2 from photosynthetic bacteria, cytochrome c-551 from pseudomonads and cytochrome c-550 from Paracoccus denitrificans.

Class II cytochromes are generally high spin but low spin members are known - eg cytochrome c-556 from Agrobacterium. These cytochromes have a single c-type

haem covalently bound near the c-terminus. The high spin members have histidine as their only protein moiety haem ligand. The low spin members have histidine as 5th ligand and methionine as 6th ligand. Their redox potentials are usually in the range -10 to +150 MV. Examples of high spin cytochromes are the cytochromes c' from photosynthetic bacteria (Pettigrew and Moore, 1987).

Class III cytochromes are low spin cytochromes having histidine-histidine ligation of the haem iron and are multihaem proteins, typically with four haems (eg cytochrome c_3 from Desulfovibrio). The redox potentials are generally in the range -100 to -300mV.

Class IV contains the c-type cytochromes which have, in addition to a c-type haem, another prosthetic group. For example the Pseudomonas cytochrome cd_1, containing a non-covalently bound d_1 group, is a member of this class. The members of this class are often membrane bound and hence poorly characterised. As more information (mainly sequence and structural information) becomes available it is likely that the cytochromes of this class will be placed into one of the better defined classes.

Section II - Functional Aspects of c-type Cytochromes

A. Mitochondrial cytochrome c

The cytochrome c from the mitochondrion represents the most fully understood cytochrome with respect to function and mechanism of action. The most important information to discover is the immediate redox partners of the cytochrome c, thus enabling the sequence of the terminal region of the electron transfer chain to be elucidated. This has been achieved for the mitochondrial electron transfer chain (see Figure 4). This stems from the relative simplicity of the mitochondrial electron transfer chain, which possesses only one cytochrome c oxidase (cytochrome aa_3), one cytochrome c reductase (cytochrome bc_1) and only one soluble c-type cytochrome. The interaction of cytochrome c with its redox partners has been extensively studied using whole mitochondrial systems or segments of the system. Examples of the systems investigated include the whole mitochondrion (Schneider and Hogeboom, 1950), cytochrome c-depleted mitochondria (Jacobs and Sanadi, 1960), the cytochrome bc_1 complex (complex III) (Rieske, 1976) cytochrome c_1 (Trumpower and Katki, 1975) and cytochrome aa_3 (cytochrome c oxidase (Okunuki et al, 1958).

Kinetic studies have played a large part in determination of the redox partners of cytochrome c - eg

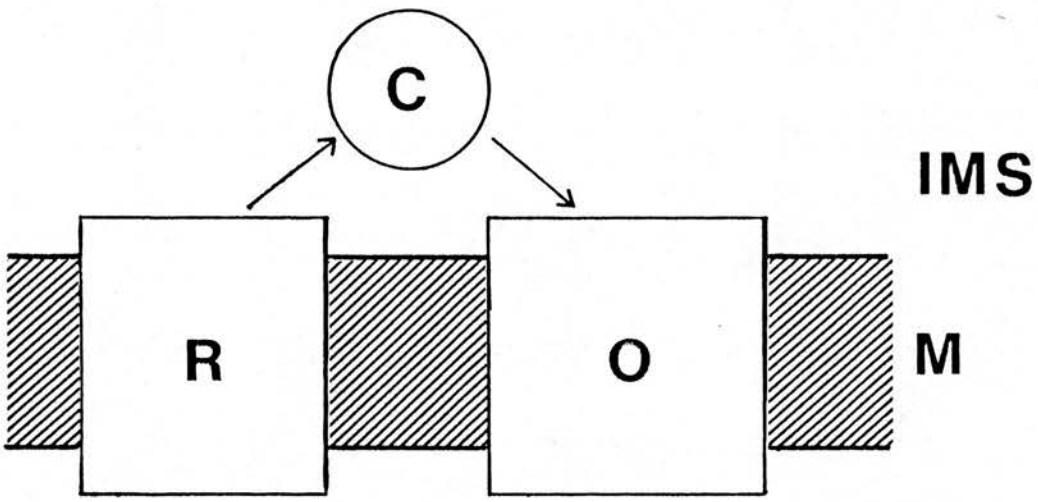
the demonstration that cytochrome bc_1 can reduce cytochrome c at a rate which is comparable to the rate of electron transfer in whole mitochondria. This may be done using steady state kinetics or stopped flow kinetics. For example, the reaction of mitochondrial cytochrome c with bovine cytochrome oxidase has been studied using steady state kinetics which resulted in the identification of two binding sites with high and low affinity. However, transient kinetics were consistent with only a single binding site (Antalis and Palmer, 1982).

Ideally, as many tests as possible should be carried out to demonstrate the redox partners. Further testing may include use of antibodies to block transfer, photolabelling the cytochrome c and on exposure to light find out which proteins the cytochrome c becomes attached to.

The reaction of mitochondrial cytochrome c with its redox partners has been taken further. Studies have mapped the mitochondrial cytochrome c - cytochrome c oxidase interaction domain through chemical modification of specific lysine residues. From knowledge of the 3D structure of the cytochrome c (Dickerson et al, 1971) five lysine residues surrounding the haem crevice have been implicated to varying degrees in binding cytochrome c oxidase (Rieder and Bosshard, 1980. Staudenmayer et al, 1977). Similar importance was assigned to these five lysine residues for interaction of mitochondrial cytochrome c with the cytochrome bc_1 complex (Smith et

Figure 4 : The Terminal Region of the Mitochondrial Electron Transfer chain

The soluble cytochrome c (C), which is found in the mitochondrial intermembrane space (IMS), has been shown to transfer electrons between a membrane (M) bound reductase (R) and a membrane bound oxidase (O).


C
IMS
R
O
M

al, 1980. Speck et al, 1979) and more specifically with purified cytochrome c_1 (Konig et al, 1980).

In addition studies have been made using computer simulations to demonstrate the interaction of mitochondrial cytochrome c with yeast cytochrome c peroxidase (and also with cytochrome b_5). It is not possible to study the interaction with cytochrome c's membrane bound redox partners since they are not available in crystalline form. These simulations were used to demonstrate how electron transfer may occur by showing that during interaction the haems were coplanar and their haem edges were approximately 8Å apart in the case of the cytochrome c:cytochrome b_5 complex (Salemme et al, 1976), and 17Å apart in the case of the cytochrome c:peroxidase complex (Poulos and Kraut, 1980).

These experimental approaches have been mentioned to demonstrate how much is known about mitochondrial cytochrome c, while very little is actually known about the role of cytochromes in bacterial systems.

B. Bacterial respiratory systems

The role c-type cytochromes play in bacterial respiration is poorly understood. One possible reason for this is the number of c-type cytochromes that can be found in the one bacterial species (seven or more is not unknown). It is often the case that more is known about the properties of the c-type cytochromes than where they fit into the electron transfer chains. For instance the amino acid sequence (Ambler and Wynn, 1973) and the X-Ray structure (Matsuura et al, 1982) have been determined for

cytochrome c-551 from Pseudomonas aeruginosa while little is known of its actual function. Similarly for cytochrome c_4, the amino acid sequence for Pseudomonas aeruginosa c_4 has been elucidated (Ambler, 1980) and a preliminary X-ray density map for the same protein has been produced (Sawyer et al, 1981). But nothing is known of the role cytochrome c_4 plays in bacterial respiration.

The scarcity of functional information on bacterial c-type cytochromes stems not only from the multiplicity of cytochromes, but also because bacteria can grow under different environmental conditions which may require alternative electron transport components. Firstly, consider the mitochondrial electron transfer chain with its one soluble and well defined oxidase and reductase, as described above. Since the environment is constant the composition of the electron transfer chain remains constant, which is not the case for bacteria. The different environmental conditions will be dealt with as two groups (1) reactions donating electrons to the cytochrome c and (2) reactions accepting electrons from the cytochrome c.

Many of the substrates which are utilised will pass electrons through the electron transfer chain via complex III and onto the terminal acceptor. Some changes of substrate may utilise the same electron transfer chain and therefore no change of electron transfer components will be observed. Some changes of substrate may involve periplasmic (soluble) enzymes which will donate electrons directly to cytochrome c. These substrates may be

organic or inorganic. For example, methanol (by Methylotrophs), hydroxylamine (Nitrosomonas), sulphide (Thiobacillus), nitrite (Nitrobacter) and ferrous iron (Thiobacillus) (Pettigrew and Moore, 1987).

Methanol dehydrogenase of Methylophilus methylotrophus is a periplasmic enzyme donating directly to cytochrome c (see Figure 5). In such a case it would be important to examine the soluble cytochromes c under different growth conditions. If other c-type cytochromes appear it may be that there are different cytochromes required for each set of enzymes. If more than one is induced it may become difficult to prove which c-type cytochrome interacts with which enzyme. (This will be discussed more fully later in this section).

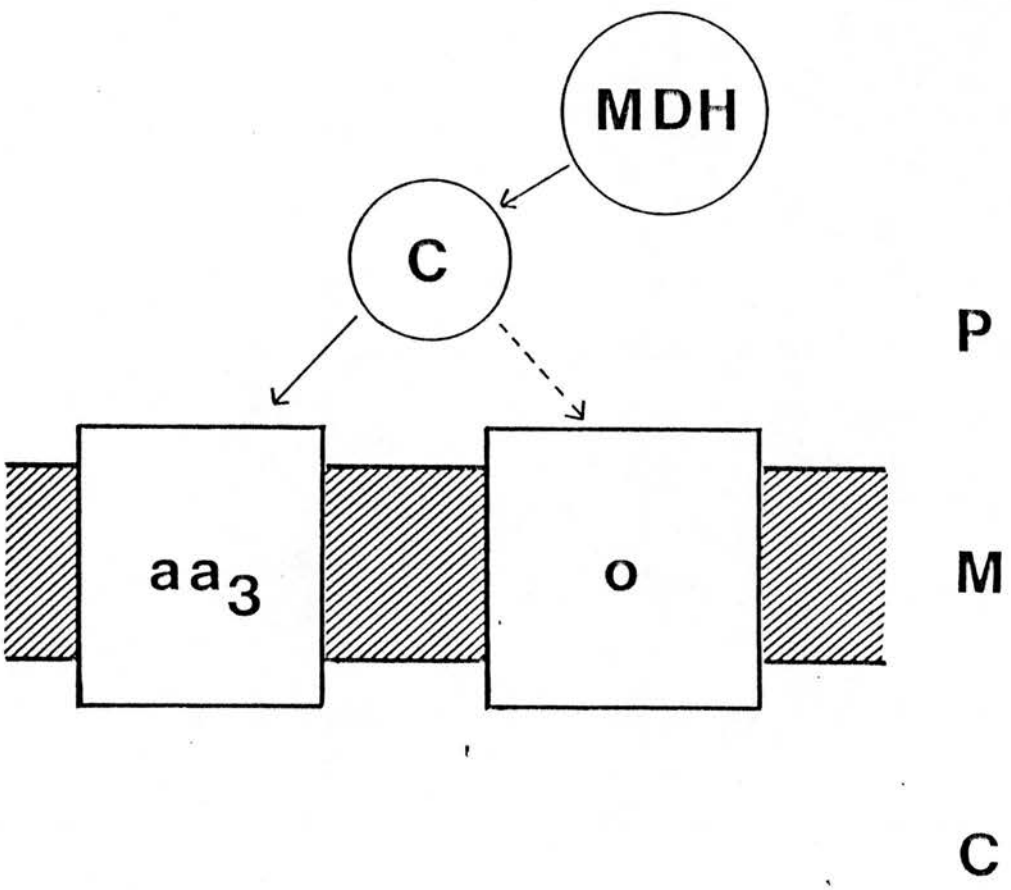
In addition to diversity in the nature of donor reactions to cytochome c there is also a range of terminal acceptors which may be utilised depending on the environmental conditions. These include both aerobic and anaerobic acceptors.

(i) Aerobic ; The aerobic terminal electron acceptors are the membrane bound cytochrome c oxidases of which four types are found - cytochromes aa_3, o, d and a_1.

It would appear to be the exception rather than the rule for bacteria to possess only one oxidase. It is more likely for two or even three of these oxidases to be present at the same time. The combination and relative quantities of the individual cytochrome oxidases will

Figure 5 : Terminal Electron Transfer Components of Methylophilus methylotrophus

Methanol is oxidised to formaldehyde by the enzyme methanol dehydrogenase (MDH), which then transfers the electrons directly to cytochrome c. Both the MDH and cytochrome c are located in the periplasm (P). The membrane bound oxidase utilised will depend on the availability of oxygen. Transfer to cytochrome o ------ occurs during oxygen limitation while during methanol limitation/ oxygen unlimited electrons will be transferred via a membrane bound reductase to cytochrome aa_3 ——— (Carver and Jones, 1983).


MDH
C
aa3
o
P
M
C

depend on the environmental conditions. For example Methylophilus methylotrophus contains both cytochromes aa_3 and o (see Figure 5). Cytochrome aa_3 is present only when methanol is limited (oxygen unlimited) but during methanol excess and oxygen limitation cytochrome o (which has a higher affinity for oxygen) is the terminal oxidase utilised (Carver and Jones, 1983). In the case of Azotobacter vinelandii cytochromes o and d are the oxidases found (see Figure 6). Under fully aerobic conditions the electrons are transferred to oxygen via the highly active cytochrome d pathway (this oxidase does not include an energy conserving site) but under low oxygen the cytochrome o (which is energy conserving) contribution is increased (Jones and Redfearn, 1967).

(ii) Anaerobic : In addition to the various combinations of aerobic acceptors there is also a range of terminal acceptors which are used in the absence of oxygen. Cytochrome cd_1 (nitrite reductase) from Pseudomonas aeruginosa is an example of such a terminal acceptor which has been shown to accept electrons from the soluble cytochrome c-551 (Barber et al, 1976).

As an example of the complexity of the terminal region of a bacterial electron transfer chain, Figure 7 shows a tentative electron transfer pathway in Pseudomonas stutzeri when grown on nitrate. Denitrification occurs only in the absence of oxygen, even at low levels of oxygen denitrification is switched off and aerobic respiration occurs. (Alefounder et al, (1983).

Figure 6 : The Branched Electron Transfer Chain of Azotobacter vinelandii

Cytochromes o and d are the terminal oxidases. The large arrow from cytochrome d to oxygen represents the large contribution of cytochrome d when oxygen is not limited (Jones and Redfearn, 1967).

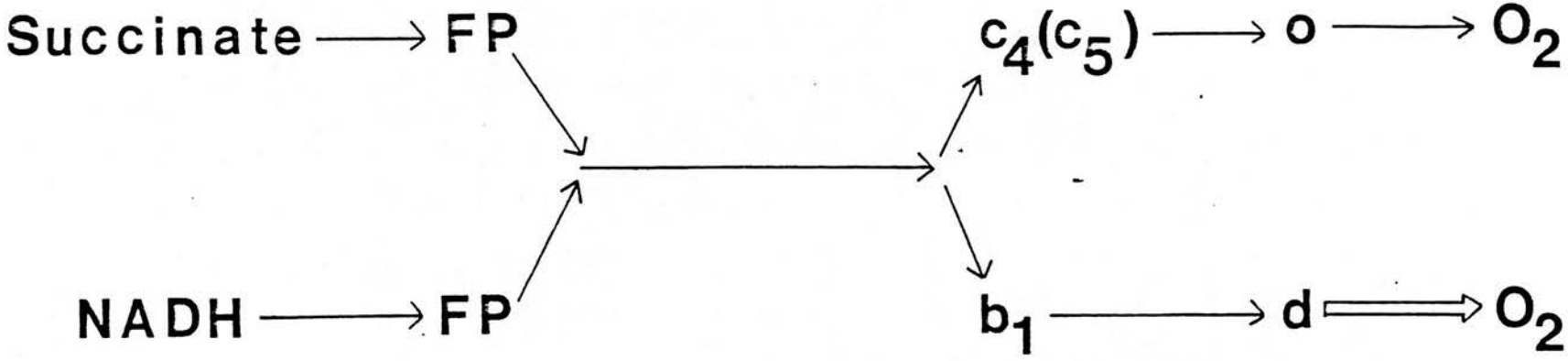

Succinate
FP
NADH
FP
$c_4(c_5)$
o
O_2
b_1
d
O_2

Figure 7 : Electron Transfer in Denitrifying Pseudomonas stutzeri

Shows a tentative scheme for electron transfer. Dotted lines show possible routes of electron transport while solid lines show actual electron transfer pathways. The 30K and cytochrome c_5 are poorly characterised proteins and are shown as broken circles (Pettigrew and Moore, 1987)

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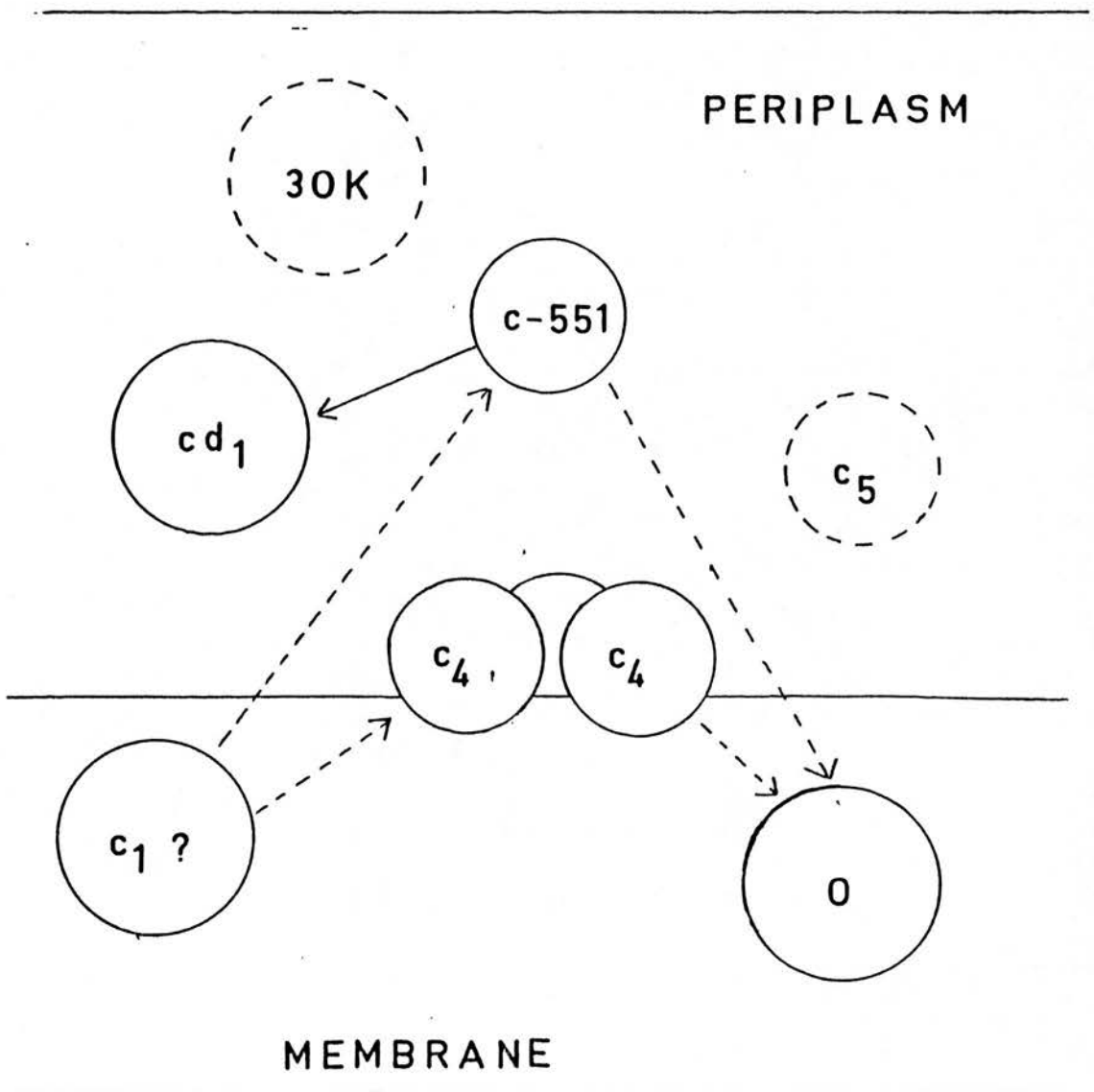

PERIPLASM
30K
c-551
cd1
c5
c4
c4
c1 ?
O
MEMBRANE

Figure 7 demonstrates the difficulties in trying to find out which cytochrome donates to which acceptor. To demonstrate electron pathways it is usual to purify the components and demonstrate the ability to exchange electrons. However, this can be risky since two proteins which exchange electrons in vitro may not actually interact in the intact system and thus cannot transfer electrons. Therefore, for systems such as these, further evidence, which may or may not be direct evidence, is required and to this end a six point strategy for determining the function of a cytochrome has been worked out.

(a) Distribution : This involves identifying the cytochrome of interest in a range of bacterial species and trying to correlate it with a particular type of respiration. For example cytochrome c_4 has been found in several Pseudomonads and also in an Alcaligenes when grown under denitrifying conditions. This may lead to the assumption that cytochrome c_4 is involved in denitrification. However, it is also present in the strict aerobe Azotobacter vinelandii (Tissieres, 1956) therefore it cannot have unique role in denitrification. Thus some other common denominator must be found. In the case of cytochrome c_4 the common factor may be cytochrome o (See this Chapter Section III). The example of cytochrome c_4 shows that this method is not fail-safe but that it may provide a starting point for subsequent studies.

(b) Induction : Having identified a possible link with a given type of respiration, the next stage would be to examine induction - ie does the cytochrome of interest appear only when one set of growth conditions prevail. For example, the Pseudomonads can grow either aerobically or anaerobically (utilising nitrate as terminal acceptor). The experimental strategy would be to compare the cytochrome complement of both aerobic and anaerobic cells and identify any changes. For Pseudomonas aeruginosa it can be seen that cytochrome cd_1 is present when the cells are grown in nitrate medium, but absent in aerobically grown cells thus implying that cytochrome cd_1 plays a unique role in denitrification. However, all cases are not as clear cut. For example, the levels of cytochrome c-551 are increased in denitrifying cells but it is also present under aerobic conditions (Parr et al, 1976).

(c) Cellular location : Acording to the hypothesis of Wood (1978) c-type cytochromes may be found attached to the periplasmic side of the membrane or in the periplasm. Therefore, for the cytochrome of interest the location must be identified.

(d) Association : During the course of purification does the cytochrome of interest co-purify with some other electron transport protein. For example, co-purification with one of the electron transport complexes may indicate that the cytochrome functions within that complex. However, the cytochrome may fortuitously co-purify with the complex and still have no function within it.

(e) Redox titration : Determination of the redox midpoint potential may give a clue as to the cytochromes position within the electron transfer chain. For instance, the more positive the potential the nearer the terminal acceptor it is likely to be located. If the cytochrome has more than one haem, redox potentiometry may reveal that the haems have the same midpoint potential or different potentials. In the case of multihaem cytochromes intramolecular electron transfer may occur which might be identified by stopped-flow kinetics. The effect of pH on the redox potential should also be examined as this may reveal possible proton pumping capabilities of a membrane cytochrome.

(f) Electron donors and acceptors : This has been discussed in some detail earlier in the section.

To identify the role of a bacterial c-type cytochrome would require full characterisation in all the above respects.

Section III - Cytochrome c_4

The first purification and partial chacterisation of cytochrome c_4 from the strict aerobe Azotobacter vinelandii was reported by Tissieres (1956) and Tissieres and Burris (1956). Further studies on the cytochrome c_4 from Azotobacter vinelandii have been reported (Swank and Burris, 1969. Campbell et al, 1973). Since then members of the cytochrome c_4 group have been isolated from several denitrifying bacteria : cytochrome c-552 isolated from the particulate (membrane) fraction of Pseudomonas stutzeri (Van Neil strain) (Kodama and Shidara, 1969), cytochromes c-552 from Pseudomonas aeruginosa and Pseudomonas mendocina (Ambler and Murray, 1973), cytochrome c-552 from Alcaligenes sp. (Shidara, 1981) and possibly cytochrome c-552 from the marine denitrifier Pseudomonas perfectomarinus (Liu et al, 1981).

Cytochrome c_4 is a dihaem cytochrome of approximately 20 000 molecular weight. It is characterised spectrally by an α- peak maximum in the range 550-552nm (eg 550nm and 552nm for the cytochromes c_4 from Pseudomonas stutzeri 224 and Pseudomonas aeruginosa respectively) and has a low α/β peak ratio, eg 1.21 for Pseudomonas stutzeri 224 cytochrome c_4 (Pettigrew and Brown, 1988). The amino acid sequence of cytochromes c_4 from Pseudomonas aeruginosa and Azotobacter vinelandii have been elucidated and show

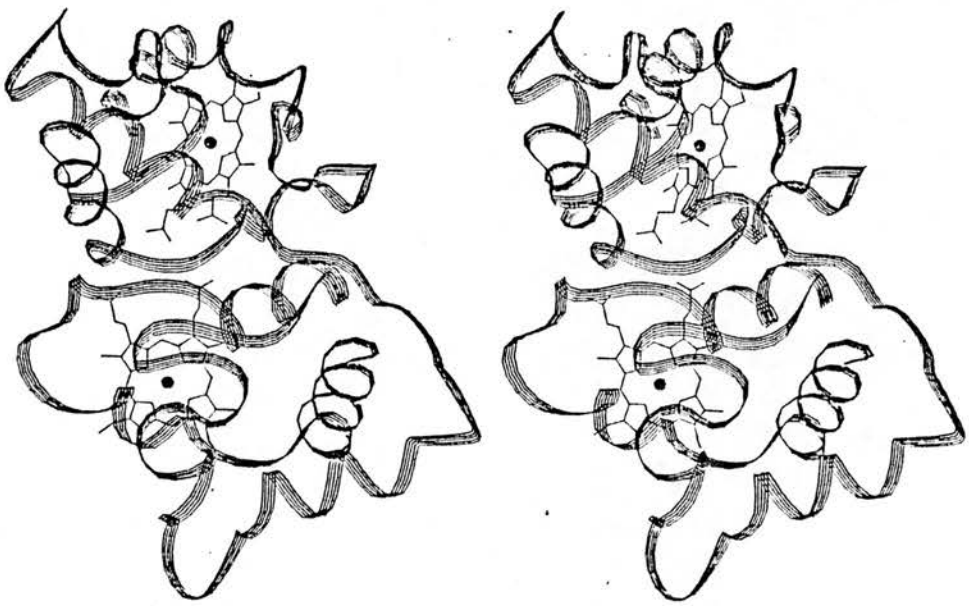
evidence of a gene duplication event (Ambler, 1982, 1984). Preliminary x-ray density maps indicate that the two halves of the protein are folded into separate domains. This is shown diagramatically in Figure 8. Each domain contains one haem and each resembling a typical monohaem c-type cytochrome (Sawyer et al, 1981).

Cytochrome c_4 is a Class I cytochrome even although it has two haems and with approximately 180 amino acid residues is much larger than a typical Class I cytochrome. The rationale for inclusion in the Class I cytochromes is as follows - (1) the sequence determinations demonstrate that cytochrome c_4 may be a product of gene duplication and has two sites for haem attachment located near the "N-terminus" of each domain; (2) the structural studies indicate that the two domains of the protein are structurally similar to a monohaem cytochrome; (3) cytochrome c_4 has a high midpoint potential, around +300 mV; and (4) the haems have histidine-methionine as 5th and 6th co-ordinating ligands.

The purification of cytochrome c_4 from several denitrifying bacteria led to a suggested correlation between cytochrome c_4 and the denitrifying process. However, this suggested correlation between cytochrome c_4 and denitrification is complicated by the presence of cytochrome c_4 in the strict aerobe Azotobacter vinelandii where it is thought to form a terminal complex with cytochrome o (Yang et al, 1979). Jurtshuk et al (1981) have reported the purification of cytochrome o from

Figure 8 : Ribbon Drawing of Cytochrome c_4 from Psedomonas aeruginosa

Tentative model for the structure of cytochrome c_4 (Courtesy of L. Sawyer, University of Edinburgh). This drawing shows a stereo pair.



Azotobacter vinelandii and found it to be composed of both a b-type cytochrome (cytochrome o) and a c-type cytochrome. It is claimed that the c-type cytochrome is cytochrome c_4. However, the c-type cytochrome has not been characterised and compared with purified cytochrome c_4. Therefore, it cannot be stated whether it is cytochrome c_4 or not.

The association of c-type cytochromes with cytochrome o may be widespread. Cytochromes o with an associated c-type cytochrome have been purified from several bacteria. For example Rhodopseudomonas palustris (King and Drews, 1976), Rhodopseudomonas capsulata (Hudig and Drews, 1983), Methylophilus methylotrophus (Froud and Anthony, 1984) and Pseudomonas aeruginosa (Matsushita et al, 1982).

Kodama (1970) noted that the levels of particulate cytochrome c-552 were elevated when Pseudomonas stutzeri (Van Neil) was grown under low aeration in the absence of nitrate or nitrite. This may reflect induction of cytochrome o as the level of oxygen is lowered.

In addition to a c-type cytochrome being associated with cytochrome o, Berry and Trumpower (1985) reported the purification of cytochrome aa_3 from Paracoccus denitrificans which had an associated c-type cytochrome. This c-type cytochrome has an apparent molecular weight of 22 000 and an α-peak maximum of 552nm (ie similar to cytochrome c_4) but no further characterisation was carried out. The ubiquinol oxidase complex, which consists of the cytochrome bc_1 and cytochrome aa_3 plus

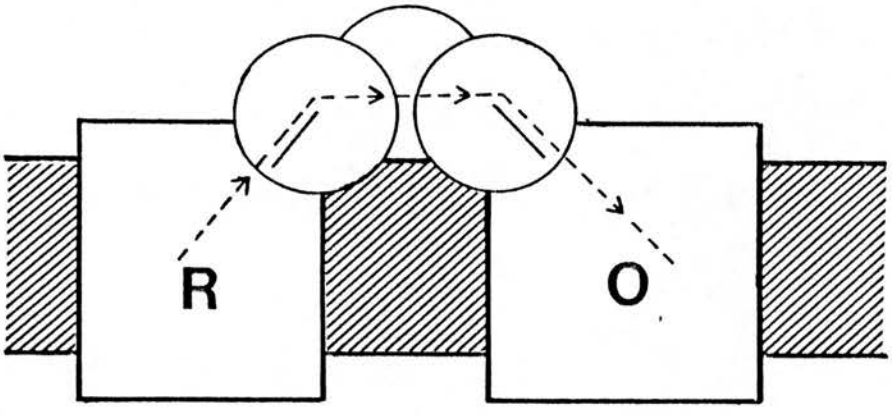
this additional c-type cytochrome, does not rely on added cytochrome c (soluble) to effect transfer through to oxygen. The suggestion is that the associated c-type cytochrome is providing a direct link between the cytochrome bc_1 complex and the cytochrome aa_3.

Daldal et al (1986) demonstrated that soluble cytochrome c_2 is not essential for photosynthesis to occur in Rhodopseudomonas sphaeroides since mutants exist which do not synthesise cytochrome c_2. However, the rates of electron transport are not as high as those reported when cytochrome c_2 was present. These results again point to some other pathway for electron transfer to the reaction centres. Takimaya et al (1982) reported the purification of the reaction centre from Rhodopseudomonas sphaeroides and demonstrated the presence of a 24 000 molecular weight c-type cytochrome associated with the reaction centre. It may be that this c-type cytochrome is involved in the transfer of electrons from the cytochrome bc_1 complex to the oxidised reaction centre without the involvement of soluble c-type cytochromes.

The dihaem, two domain nature of cytochrome c_4 may be able to interact with both a membrane bound reductase and a membrane bound oxidase simultaneously, shown diagramatically in Figure 9. Thus cytochrome c_4 would provide a direct link for electron transfer between the cytochrome bc_1 complex and the membrane bound terminal oxidase which in Pseudomonas stutzeri is likely to be cytochrome o. Work is currently in progress to

Figure 9 : Electron Transfer from Reductase to Oxidase Through Cytochrome c_4

(The broken line shows a tentative electron transfer scheme.) Electrons are transferred to one haem of cytochrome c_4 then by intramolecular transfer to the other haem and then to the oxidase.



P

M

C

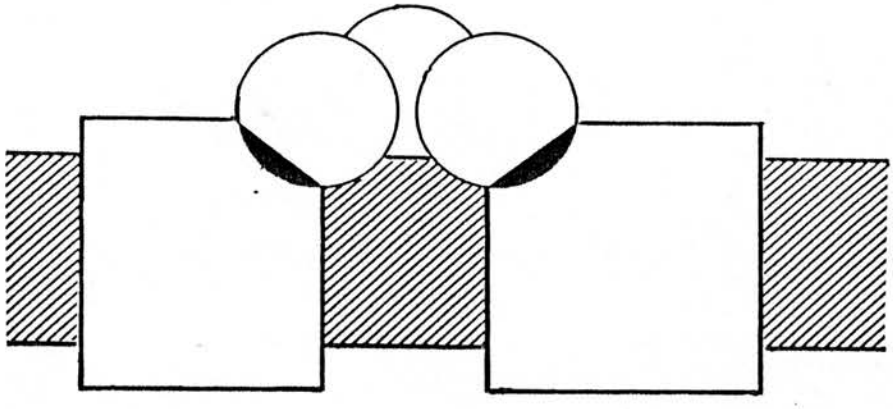
demonstrate the association of cytochrome c_4 with the cytochrome o (as suggested by Yang et al, 1979). The mechanism of electron transfer outlined in Figure requires that the electron is transferred from the bc_1 complex to one of the haems of cytochrome c_4. The next stage would require an intramolecular transfer of the electron to the other haem before passage to the oxidase. Currently, Sykes and co-workers at the University of Newcastle are studying the kinetics of reduction of cytochrome c_4 and these studies should demonstrate if intramolecular transfer is occurring. It would also be interesting to find out if elecron transfer occurs in the absence of soluble c-type cytochromes in Pseudomonas stutzeri.

The nature of attachment of cytochrome c_4 to its redox partners would appear to be through hydrophobic interaction since butanol is required to solubilise it. It is interesting to note that even although cytochrome c_4 is attached to its redox partner by hydrophobic interaction, it is also soluble in aqueous buffers. It is suggested that cytochrome c_4 'changes conformation on extraction into the aqueous phase, which is shown diagramatically in Figure 10. The properties of this soluble form may be different from the membrane bound form, but as yet no studies of cytochrome c_4 have been carried out when it is still attached to the membrane.

Figure 10 : Diagramatic Representation of the Hypothetical Conformation of Cytochrome c_4 in its Membrane Bound and Soluble Forms

The darkened areas represent hydrophobic areas. (a) shows the membrane bound form; the hydrophobic areas on the cytochrome c_4 interact with complementary hydrophobic areas on its redox partners. The remainder of the cytochrome c_4 molecule is exposed to the aqueous periplasm (P) and therefore the surface amino acids are likely to be polar. (b) in aqueous buffer the conformation is altered such that the hydrophobic faces of each domain interact thus presenting only polar groups to the solution.

(a)

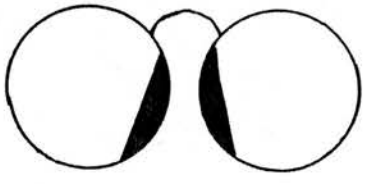


P

M

C

(b)



CHAPTER II : MATERIALS AND METHODS

MATERIALS

Sephadexes G-25 (coarse, fine and superfine) and G-75 (superfine) were obtained from Pharmacia (Great Britain) Ltd. Middlesex. CM cellulose (CM52) and DEAE cellulose (DE52) were obtained from Whatman Ltd. Kent. Acrylamide and bis-acrylamide were purchased from BDH Ltd. Dorset. Tetramethylbenzidine (TMBZ), was purchased from the Sigma Chemical Co. These, and all other chemicals, were of the best grade available.

METHODS

Pseudomonas stutzeri

Pseudomonas stutzeri 224 (ATCC 17591) was first isolated from a clinical specimen (urine) and a taxonomic study was carried out by Stanier et al (1966). Further studies (Mandel, 1966) on several species of Pseudomonas stutzeri revealed, on the basis of the C+G content, that they could be separated into two distinct groups termed Pseudomonas stutzei (G+C content of 66-64%) and Pseudomonas 'stanieri' . Pseudomonas stutzeri 224 (the bacterium used in this study is classified in the low G+C group, Pseudomonas 'stanieri'.

On the basis of the G+C content and more than 100 nutritional and general characteristics Pseudomonas perfectomarinus has been reclassified as Pseudomonas stutzeri. The G+C content of 62% places Pseudomonas perfectomarinus (ATCC 14405) into the 'stanieri' group, ie the same as Pseudomonas stutzeri 224.

It is therefore important to consider the study of Liu et al (1983) in comparison with the work described in this thesis. These workers reported the comparison of cytochromes from aerobically and anaerobically grown Pseudomonas perfectomarinus (ATCC 14405). Of importance to this thesis is the reported absence of cytochrome c_4

from Pseudomonas perfectomarinus. It should be noted that cytochromes were only purified from the soluble fraction by Liu et al (1983) and no attempt was made to purify and characterise the membrane cytochromes. This thesis and a study by Pettigrew and Brown (1988) demonstrated that cytochrome c_4 is predominantly membrane bound. It was also noted by Pettigrew and Brown (1988) that no soluble cytochrome c_4 was detected in aerobically grown Pseudomonas aeruginosa and in addition only small amounts (ie approx. 3% of the total) of cytochrome c_4 was found in the soluble fraction of aerobic cells. This compares with 15% (aerobic) and 35% (nitrate) of the total cytochrome c_4 being soluble in Pseudomonas stutzeri 224. It is conceivable that cytochrome c_4 may be totally membrane bound in both aerobic and anaerobic Psuedomonas perfectomarinus. Therefore comparisons between the two species at this stage cannot be made with regards to cytochrome c_4 distribution. Further studies would be required on the membrane bound cytochromes of Pseudomonas perfectomarinus for a statement on presence or absence of cytochrome c_4.

A. Growth of Bacteria

Pseudomonas stutzeri (Stanier 224, ATCC 17591) (obtained from Joan Fleming, Molecular Biology, University of Edinburgh) were grown aerobically in a medium containing 17mM tri-sodium citrate, 7mM KH_2PO_4, 2mM $MgSO_4.7H_2O$ and 4g/l yeast extract (Oxoid Ltd), the pH was adjusted to 7 by addition of 3M NaOH. Cultures were incubated for 16 hrs at 32°C with constant aeration. For denitrifying growth, cells were grown aerobically in the above medium for 5 hrs after which aeration was stopped and Na NO_3 was added to a final concentration of 58mM. Cultures were then incubated anaerobically for a further 11 hrs. Cells were grown either in a 10l fermenter, with constant bubbling with sterile air, or in 1l flasks with shaking. Cells were harvested by centrifugation (Alfa-Laval LAB 102B-05 continuous flow centrifuge).

B. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

15% SDS-polyacrylamide gels were prepared as slabs (130 x 150 x 1mm) with a 4% stacking gel and run using the buffer system of Laemmli (1970) with the addition of 2mM EDTA. Gels were either run at 180V for 4-5 hrs or at 40V overnight.

Proteins of less than 10 000 molecular weight were analysed on gels of 20% acrylamide/10% glycerol with a 10% stacking gel, as described by Cabral and Schatz (1979). These gels were run for 20 hrs at 100V.

Gels were either stained for haem using the method described by Goodhew et al (1986) or for protein using Coomassie brilliant blue R250. Gels were photographed through a yellow filter (Cokin A.001). Gels were scanned using a Shimadzu CS-930 gel scanner at 690nm for haem stained gels or 600nm for protein stained gels in transmission mode. Beam dimensions were 0.05 x 2mm and step size was 0.2mm.

C. Pyridine Ferrohaemochromes of Cytochromes

The haem c concentrations of solutions of purified cytochromes was calculated from the pyridine ferrohaemochrome spectra using a millimolar extinction coefficient of 31.18 at the α-peak (550nm) for the pyridine ferrohaemochrome (Bartsch,1971). This calculated concentration was then used to estimate the millimolar extinction coefficients of the α- and β-peaks from the native spectra (recorded in 0.1M phosphate, pH 7). Spectra were recorded using a Cary 219 spectrophotometer.

D. Redox Potentiometry

Oxidative and reductive titrations were carried out in an anaerobic cuvette. Anaerobiosis was achieved by bubbling the cuvette contents with argon for 15 mins prior to the titration and maintained by constantly bubbling throughout the titration. The cuvette contents were constantly stirred using a magnetically driven cell stirrer (Bel-Art).

Typically the cuvette contained approximately 5 M cytochrome in 20mM sodium phosphate pH 7, 20μM phenazine ethosulphate (PES, Sigma) 20 M phenazine methosulphate (PMS, Sigma), 20 μM diaminodurol (DAD, Aldrich), 20 μM ferric ammonium sulphate and 0.4mM EDTA. Total volume was 3ml.

The ambient potential was monitored by a Pt. pin electrode in combination with an Ag/AgCl reference (Russell pH Ltd., Auchtermuchty, UK). The potential with reference to the hydrogen electrode (E_h) was obtained by adding 198mV (the standard potential of the Ag/AgCl electrode (Bates, 1954)) to the measured ambient potential.

Oxidative and reductive titrations were carried out by injection of μl quantities of potassium ferricyanide (approx. 15 mg/ml) and sodium dithionite (approx 8 mg/ml in 0.1M phosphate pH 7). After each addition the state of reduction was recorded spectrophotometrically (Pye Unicam SP 1800 spectrophotometer) and the ambient potential noted. The pH of the cuvette contents was measured. Full reduction was achieved by the addition of solid sodium dithionite.

E. Protein Estimation

Protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as standard.

F. Amino Acid Analysis

Hydrolysis of samples containing 5-10 nmoles protein was carried out in vacuo in sealed tubes for 20 hrs at 105^{o}C in 6 N HCl (BDH, 'Aristar') plus 0.1% phenol. Samples were then dried and subsequently redissolved in sodium citrate buffer, pH 2.2. Samples were analysed by the method of Spackman (1963) on a Locarte analyser. The number of individual amino acid residues was calculated relative to the haem content (as measured from the pyridine ferrohaemochrome).

The haem was removed from the cytochrome (Ambler et al, 1969) and cysteine was determined as cysteic acid by

oxidation with performic acid prior to acid hydrolysis (Moore, 1963).

G. Western Blotting

Proteins were electrophoretically transferred from 15% SDS-polyacrylamide gels to nitrocellulose (Schleicher and Shuell, West Germany. 0.45 m pore size) in a Bio-Rad Electroblot cell using the method of Burnette (1981). Tween 20 (Sigma) (0.1%) and Marvel milk powder (2%) were used as blocking agents for nitrocellulose binding sites. 1/500th dilution of anti-stutzeri c_4 antibody (raised in rabbits by Dr G W Pettigrew) was used as probe. Secondary probe was donkey anti-rabbit IgG coupled to horse radish peroxidase (Scottish Antibody Production Unit). Chloronaphthol (Sigma) was used as electron donor to the horse radish peroxidase. Nitrocellulose sheets were allowed to soak in a solution of 0.5mg/ml chloronaphthol/ 2.89mM hydrogen peroxide in TBS/methanol (5:1).TBS is 25mM TRIS-HCl/0.15M NaCl, pH 7.4. Oxidation of the chloronaphthol produces a black precipitate on the nitrocellulose. The reaction was stopped by washing the nitrocellulose in distilled water. Blots were photographed using a yellow filter (Corning A.001) and scanned using a Shimadzu CS-930 gel scanner at 500nm in reflectance mode.

H. ELISA

75 μl of a 10 g/ml solution of antigen in 25 mM phosphate /0.1M NaCl (phosphate buffered saline, PBS) was dispensed into the wells of a microtitre plate (Dynatech) - ie each well receiving 0.75 g antigen. This was left overnight at 4^{o}C to allow coating of the plastic wells by non-specific adhesion. The antigen solution was discarded and the wells were washed 3 times with PBS/0.1% Tween 20 (Fresh solution was used for each wash).

The antiserum was diluted 1 in 20 in PBS + 2% BSA. 150μl of this was dispensed into the wells of column 1. Into the rest of the wells was dispensed 75μl PBS + 2% BSA. 75μl was transferred serially down the plate from column 1 to 10 giving a range of serum dilution of 1/20

to 1/10240. This was left for 30 mins at room temperature, after which the wells were washed 3 times (as above).

A 1:1000 dilution of GARIGG-AP (goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma)) was made into PBS + 2% BSA and 75μl of this was dispensed into each well. This was left for 30 mins at room temperature after which the wells were washed 3 times as described above.

A tablet of p-nitrophenyl phosphate (pnpp) (Boehringer) was dissolved in 10ml 50mM glycine NaOH, pH 10.5 containing 0.5mM $MgCl_2$ giving a substrate concentration of 5.4mM. 75μl of this was added to all the wells and incubated for 30 mins at 37^{o}C. The production of p-nitrophenol (by the action of alkaline phosphatase on pnpp) was monitored by recording the absorbance at 405nm in the microtitre plate wells in a microtitre plate reader (LKB). The results were plotted as absorbance against serum dilution.

CHAPTER III : PROPERTIES OF PSEUDOMONAS STUTZERI 224 CYTOCHROME c_4

Cytochrome c_4 was purified as described in Chapter IV.

A. Spectral properties

The spectrum shown in Figure 11 was recorded between 250nm and 580nm. The oxidised spectrum shows a Soret absorption maximum which on reduction with sodium dithionite is shifted to 415nm. The ratio of the absorbances at 550nm and 270nm is 1.31. Figure 12 shows the spectrum in the 510 to 580nm range. The solid line is a dithionite reduced spectrum of the native cytochrome c_4 and shows an α- peak maximum at 550nm and a β-peak maximum at 522nm. The ratio of the α-peak to β-peak was 1.23. This low α/β ratio is characteristic of cytochrome c_4. The pyridine ferrohaemochrome (broken line) is also shown in Figure 12, with its characteristic α-peak maximum at 550nm. Using the pyridine ferrohaemochrome the millimolar extinction coefficients of the α- and β-peaks of the native spectrum were calculated to be 22.06 and 17.94 respectively (extinctions are expressed per mole haem). The presence of histidine-methionine ligation of the haem is revealed spectrally by an absorption band at 695nm. The spectrum of oxidised cytochrome c_4 is shown in Figure 13 and an absorption band at approximately 695-700nm is noted. The absorption maximum was identified at 698nm using a more sensitive scale (not shown) and a millimolar extinction coefficient of 0.694 was calculated.

B. Mobility of cytochrome c_4 on SDS-PAGE

Figure 11 : Spectrum of Cytochrome c_4

The oxidised spectrum (broken line) and reduced spectrum (solid line) were recorded between 250nm and 580nm.

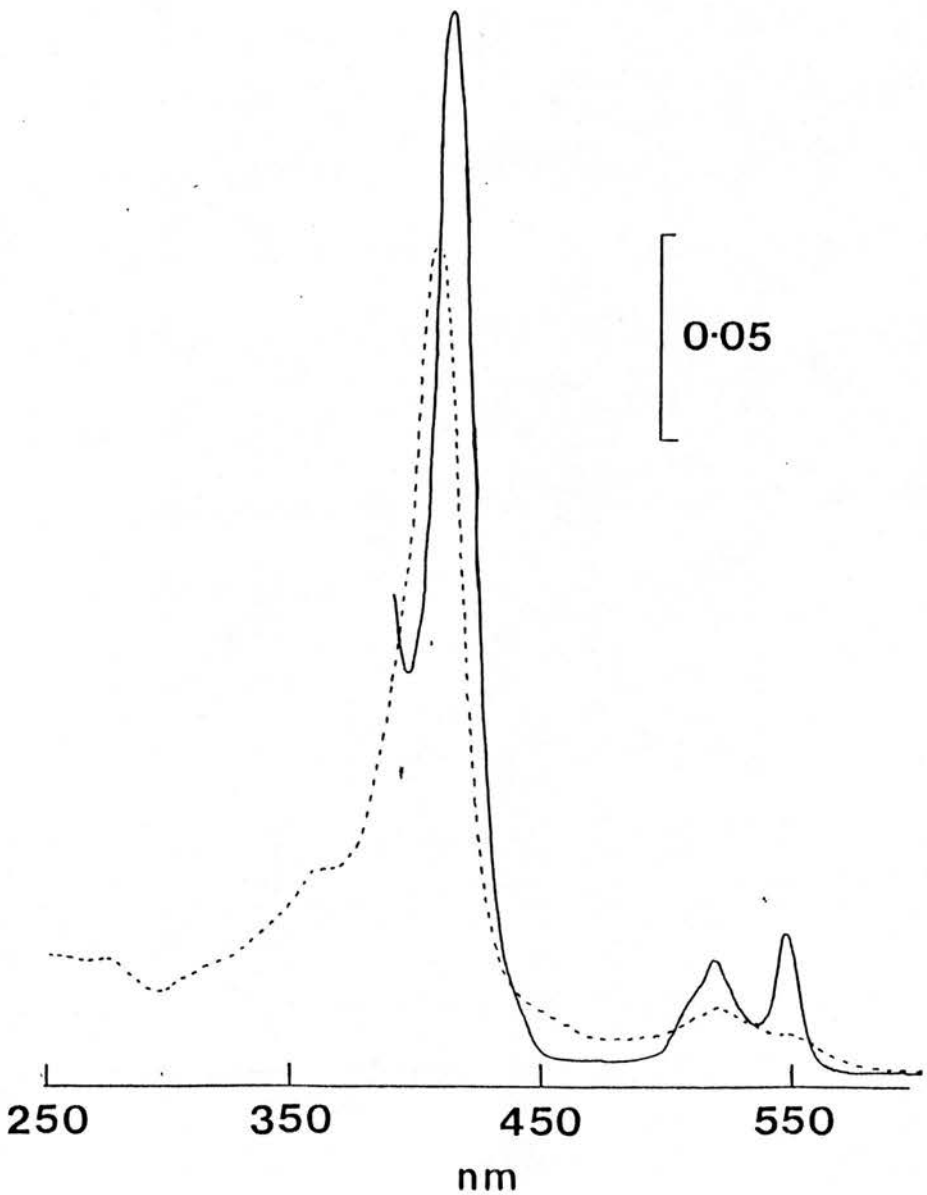

0·05
250
350
450
550
nm

Figure 12 : Spectrum of Cytochrome c_4

The dithionite reduced spectrum (solid line) was recorded between 510 and 580nm. The pyridine ferrohaemochrome (broken line) was recorded using the same cytochrome concentration as for the native spectrum.

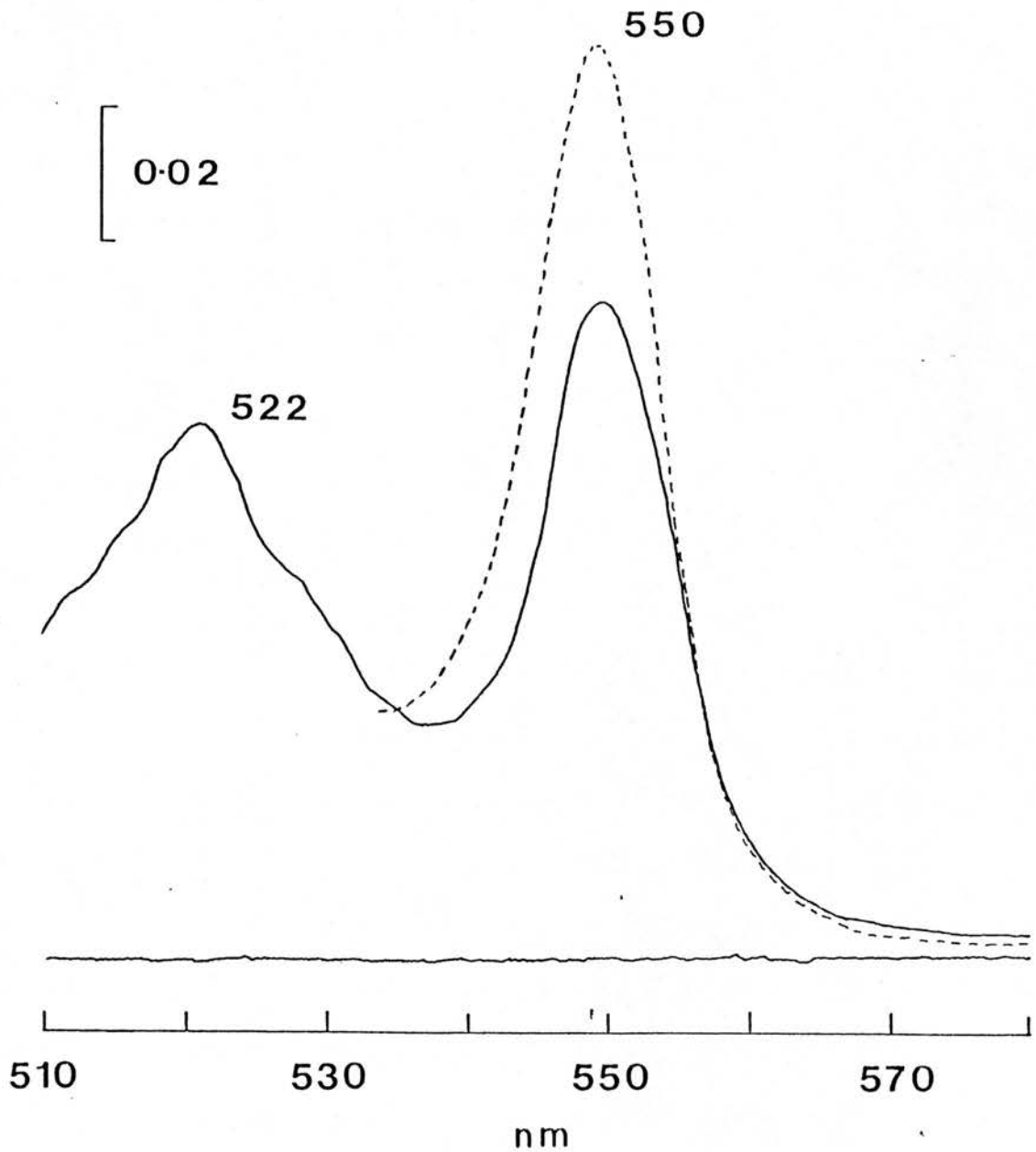
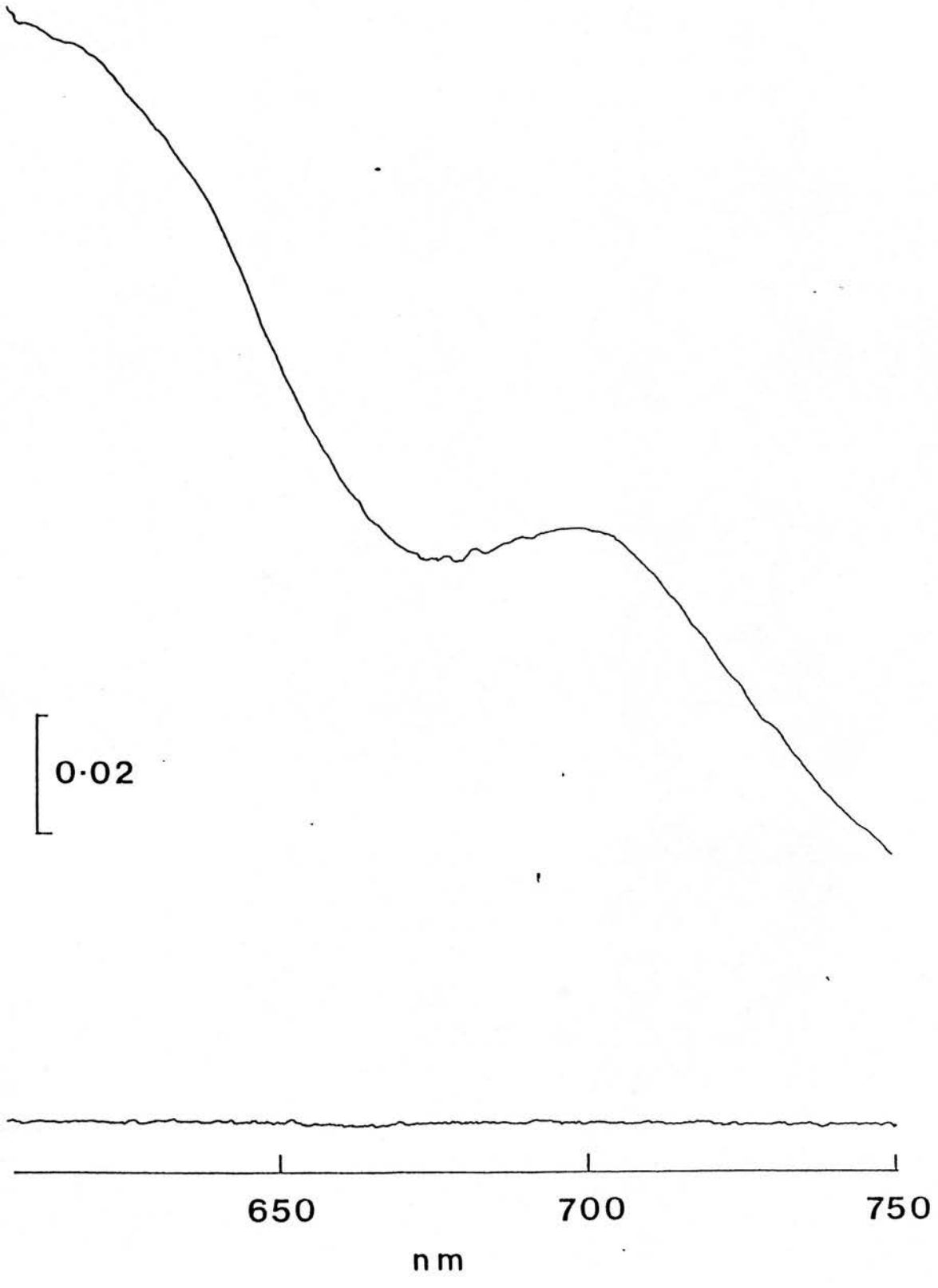

550
0·02
522
510
530
550
570
nm

Figure 13 : Investigation of the 695nm Band in Cytochrome c_4

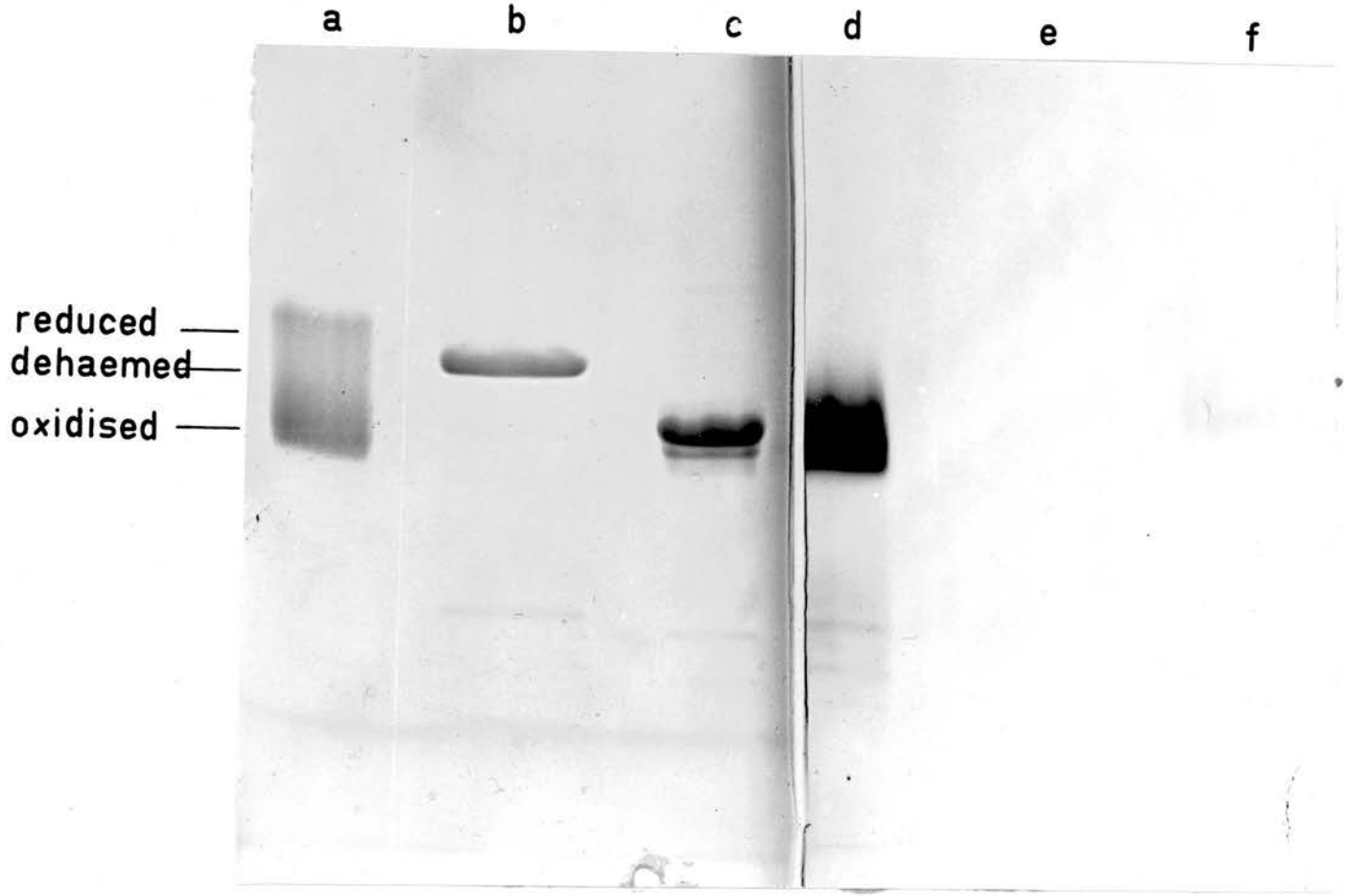

0·02
650
700
750
nm

SDS-PAGE can be used to estimate the molecular weights of proteins. However, for cytochrome c_4 three different molecular weights can be calculated depending on how the cytochrome was treated before application to the gel. Three treatments will be shown - namely (1) removal of haem, (2) addition of oxidising agent before addition of SDS, and (3) addition of reducing agent before addition of SDS.

Figure 14, lanes (a)-(c) show dithiothreitol (reducing agent) treated cytochrome c_4 (lane (a)), dehaemed cytochrome c_4 and ferricyanide (oxidising agent) treated cytochrome c_4 stained for protein. It can be seen from the gel that the oxidised c_4 and the dehaemed c_4 run as single bands but with different mobilities. Treatment with dithiothreitol produces two bands, the lower of which runs with the same mobility as oxidised c_4. The upper band runs with a mobility slower than both the oxidised and dehaemed cytochromes c_4, this slow band being 'reduced' c_4. However, care must be used when talking about oxidised and reduced cytochromes c_4. Oxidising cytochrome c_4 ensures that the iron is in the Fe^{3+} form and on denaturation of the cytochrome the Fe^{3+} remains with the cytochrome (it is this form which has peroxidase activity and can be stained for haem). Reducing the cytochrome produces the Fe^{2+} form and on denaturation the Fe^{2+} may not remain with the haem. Therefore, the reduced form after SDS treatment is probably the porphyrin form (Katan, 1976, Wood, 1981). The porphyrin form fluoresces and forms the basis of

Figure 14 : Mobility of Cytochrome c_4 on SDS-PAGE

Lanes (a)&(c) were loaded with 1.8 nmole Cytochrome c_4, lane (b) was loaded with .07 nmole dehaemed cytochrome c_4 and were stained for haem with Coomassie Blue. Lanes (d)&(f) were loaded with 0.09 nmole cytochrome c_4, lane (e) was loaded with 0.035 nmoles dehaemed cytochrome c_4 and stained for haem. Lanes (a) and (f)
were treated with dithiothreitol, lanes (b) and (e) were dehaemed and lanes (c) and (d) were treated with ferricyanide.


a
b
c
d
e
f
reduced
dehaemed
oxidised

locating cytochromes c in gels by their red fluorescence (Wood, 1981).

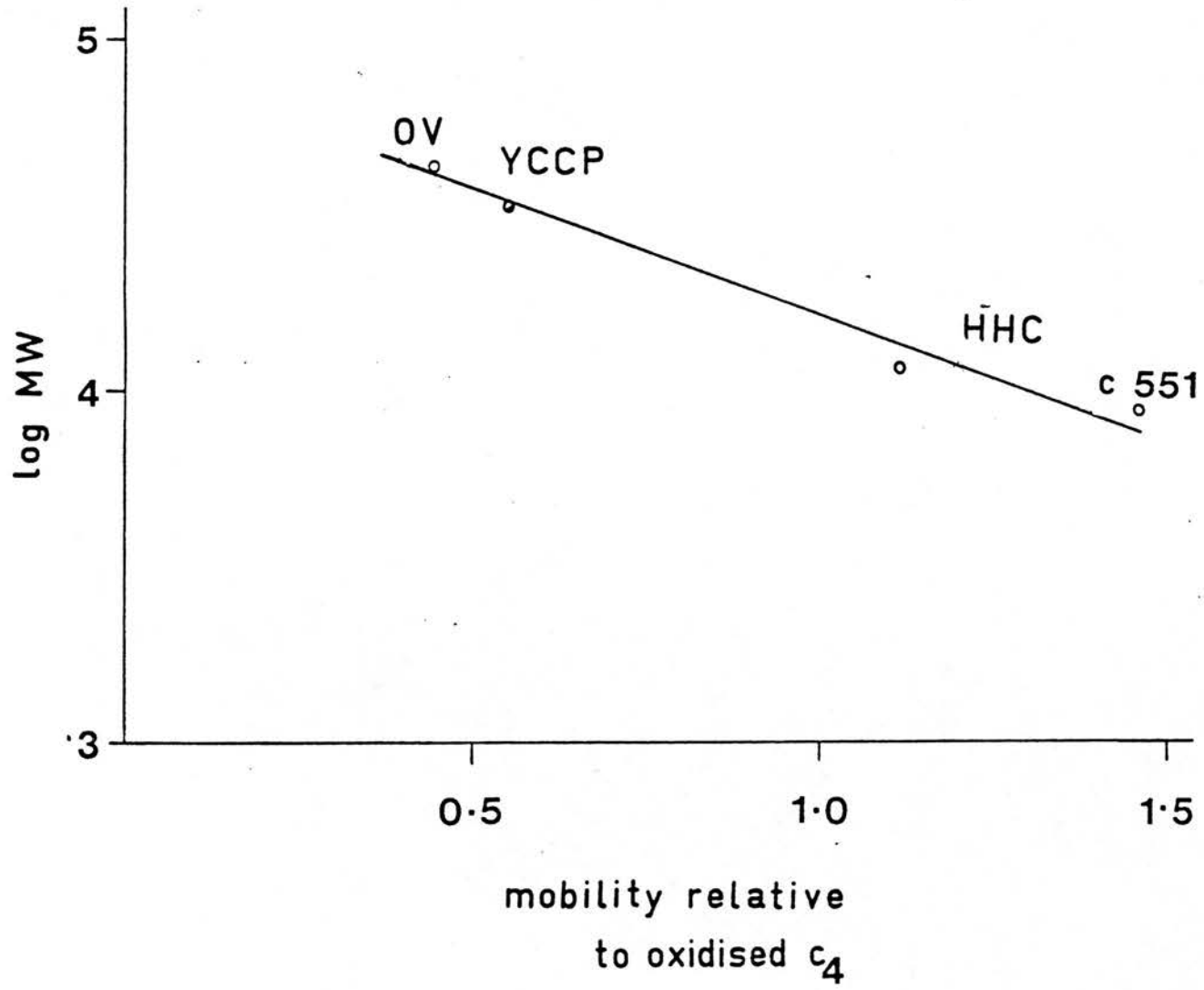
Lanes (d), (e) and (f) show oxidised, dehaemed and reduced cytochrome c_4 respectively stained for haem. Lane (d) shows one haem stained band with the same mobility as the oxidised cytochrome c_4 of the protein stained gel. The dehaemed sample, lane (e) shows no haem staining, which is not surprising since haem is required for the haem stain reaction. Lane (f), the dithiothreitol treated sample, shows only one haem stained band which runs with the same mobility as the oxidised sample (lane (d)). The slower dithiothreitol band is invisible to haem staining, the porphyrin form being unable to carry out the peroxidase reaction. The gel shows that for cytochrome c_4 (with haem attached) the mobility is dependent on the method of gel sample preparation.

For the quantitation experiments in Chapter IV it is important to ensure full oxidation of the cytochromes before addition of the SDS since any reduced cytochrome will be invisible by haem staining.

The molecular weights of the three "forms" of cytochrome c_4 were estimated from a standard plot of log molecular weight against mobility relative to the oxidised form of cytochrome c_4 (See Figure 15 for standard plot). The relative mobilities of three forms of cytochrome c_4 were calculated from the gel shown in Figure 14. Molecular weights of 16 200, 22 400 and 20 900 were obtained for oxidised (lane (c), Figure 14)

Figure 15 : Standard Curve of Log MW vs Mobility Relative to the Oxidised Form of Cytochrome c_4

The curve was constructed from an average of 2 gels. The standards used were ovalbumin (45 000), yeast cytochrome c peroxidase (34 000), horse heart cytochrome c (11 700) and *Pseudomonas aeruginosa* cytochrome c-551 (9000).


5
4
·3
log MW
OV
YCCP
HHC
c 551
0·5
1·0
1·5
mobility relative
to oxidised c_4

reduced (lane (a), Figure 14) and dehaemed (lane (b), Figure 14) respectively.

The large difference in molecular weight between the dehaemed cytochrome c_4 and oxidised cytochrome c_4 (4675) may be explained by the retention of some compact protein structure around the haem moiety, even in SDS. The loss of the iron in the reduced form allows full denaturation of the protein moiety thus allowing a better estimate of the molecular weight. It is assumed that in dehaemed cytochrome c_4 treatment with SDS produces full denaturation and thus gives the best estimate of cytochrome c_4 molecular weight. However, it should be noted that the porphyrin form (reduced) is only 1494 heavier in molecular weight - ie approximately the molecular weight of two haem moieties (1224).

C. Redox titration of cytochrome c_4

The redox properties of cytochrome c_4 will be discussed in detail in Chapter VI. However, the purification of cytochrome involves lowering the pH of the cytochrome solution to 4. It is important to find out if this lowering of pH causes any damage to the protein. This was achieved by performing redox titrations on cytochrome c_4 before and after the pH was lowered.

The redox titrations were carried out on the same batch of cytochrome c_4. The measured redox potentials of the pre-pH4 cytochrome c_4 were +300 and +195mV and post-pH4 the redox potentials were +302 and +194mV

indicating that reducing the pH to 4 has no effect on the cytochrome c_4.

D. Effect of proteases on soluble and membrane bound cytochrome c_4

Experiments were carried out using trypsin (bovine pancreas, Sigma) and subtilisin BPN (Sigma) to assess the susceptibility of the membrane bound and soluble forms of cytochrome c_4 to protease action. Membrane protein to protease ratios of 20:1, 10:1 and 5:1 (by weight) were used. Total membrane protein will overestimate the amount of protein exposed to protease since some membrane protein is buried within the lipid bilayer. Therefore the accessible membrane protein:protease ratio will actually be lower than stated. The ratios of purified cytochrome c_4:protease used were 20:1, 10:1 and 5:1. The digestion conditions used are detailed in Chapter V. At the end of the 30 mins incubation period trypsin was inhibited by addition of a 10 fold molar excess of Soyabean trypsin inhibitor (Sigma) and subtilisin by a 30 fold molar excess of PMSF. A control tube with the appropriate amount of protein but no protease was also included. The pH of the samples were noted after the incubation time and in each case was found to be unchanged at 7.8. It was important to show this since proteolysis of proteins releases protons and if the buffering capacity was not adequate the pH of the solution would fall. Since the optimum pH for these proteases is around 8 lowering the pH would lower the reaction rate. The stability of the pH noted means that the buffering capacity is sufficient.

Samples were taken after addition of the inhibitor and assessed on SDS-PAGE stained for haem. Densitometer scans yielded areas under the c_4 peak. The areas were then expressed as a percentage of the control.

Figures 16 and 17 show the results of tryptic and subtilisin digests respectively. The tryptic digest shows a dramatic difference betwen pure, soluble cytochrome c_4 and membrane bound cytochrome c_4. Even at the highest trypsin:protein ratio 73% of the membrane c_4 remains compared to 8% of the soluble c_4. This difference in susceptibility may be explained in two ways - (1) the structure of cytochrome c_4 is different when it is attached to the membrane meaning that the sites of trypsin action are no longer exposed to the protease, or (2) the protein structure remains the same but the membrane or other proteins block the site of trypsins action. Subtilisin on the other hand digests both purified cytochrome c_4 and membrane bound cytochrome c_4 suggesting sites of action are available in both membrane bound and soluble cytochrome c_4.

E. Amino acid composition

The amino acid composition of Pseudomonas stutzeri 224 cytochrome c_4 is shown in Table 1, the calculated molecular weight was calculated assuming two haems and four cysteines and is in fairly good agreement with the molecular weight calculated for the dehaemed cytochrome c_4 by SDS-PAGE.

Four cysteines were found when cytochrome c_4 was analysed for cysteic acid.

Figure 16 : Plot of Percentage Cytochrome c_4 Remaining Against Ratio of Protein : Trypsin

Assumed quantity of cytochrome c_4 at zero trypsin as 100%. Broken line represents membrane bound cytochrome c_4, solid line represents purified, soluble cytochrome c_4. 0 represents no addition of trypsin. Incubated for 30mins at $30^{o}C$.

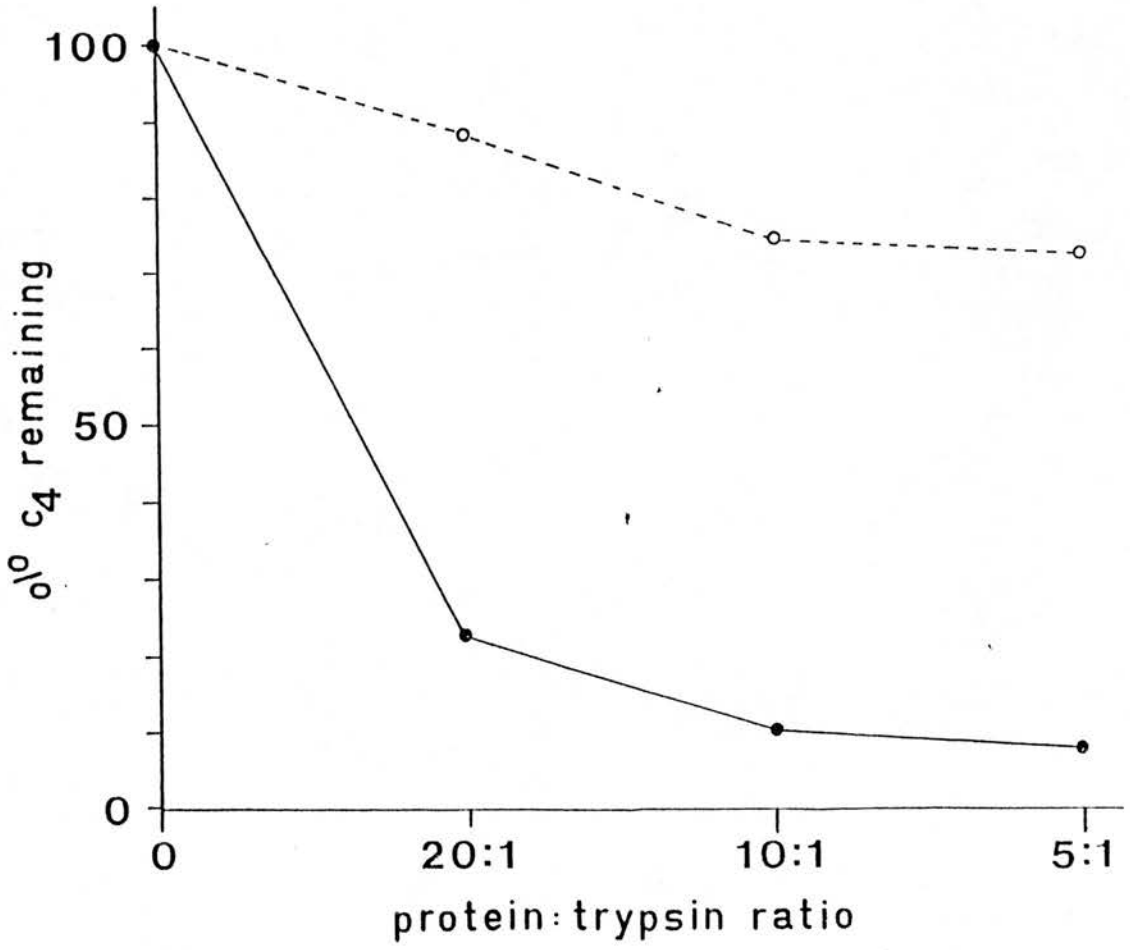

100
50
0
% C_4 remaining
0
20:1
10:1
5:1
protein: trypsin ratio

Figure 17 : Plot of Percentage Cytochrome c_4 Remaining Against Ratio of Protein : Subtilisin

Assuming quantity of cytochrome c_4 at zero subtilisin as 100%. Broken line represents membrane bound cytochrome c_4, solid line represents purified, soluble cytochrome c_4. 0 represents no addition of subtilisin. Incubated for 30mins at $30^{o}C$.

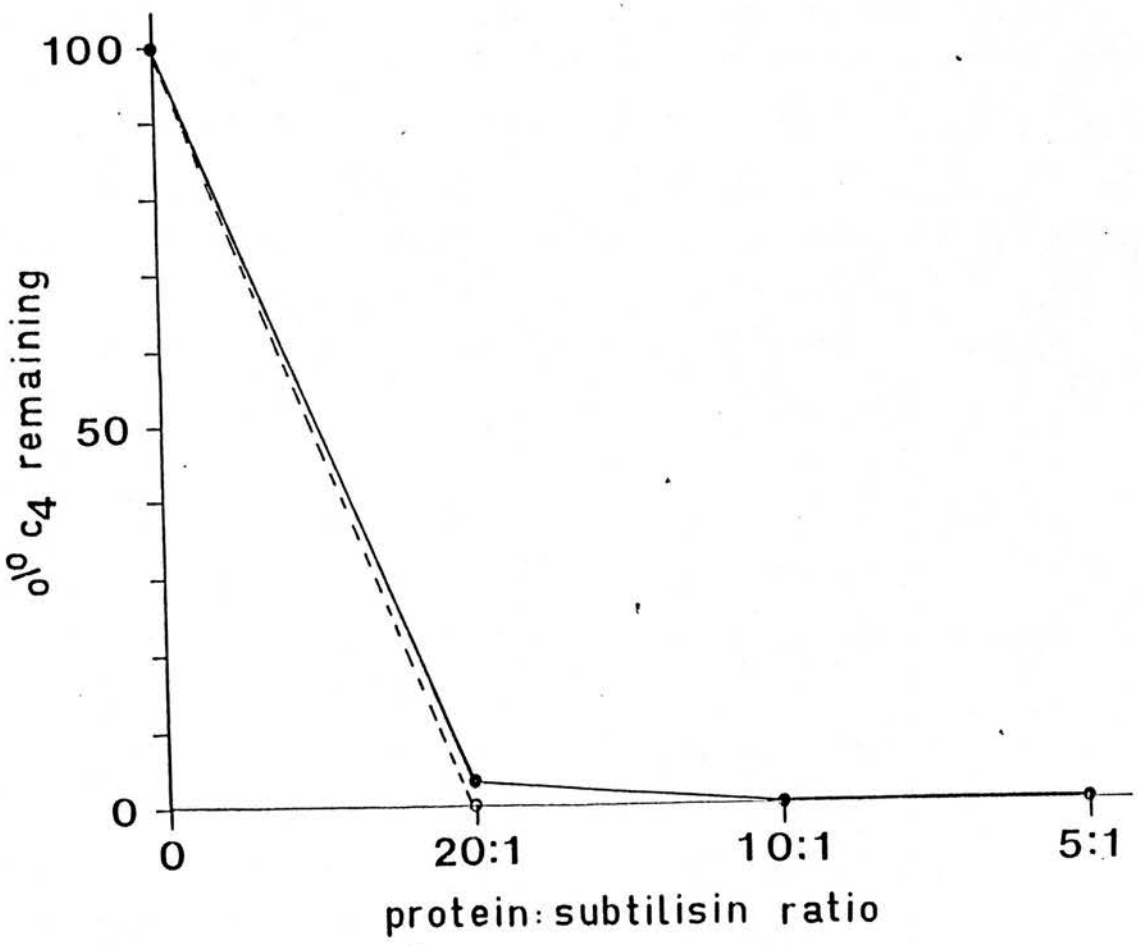

100
50
0
$_{o}I^{o}$ c_4 remaining
0
20:1
10:1
5:1
protein: subtilisin ratio

Table I : Amino Acid analysis of Pseudomonas stutzeri 224 Cytochrome c_4

The number of individual amino acids was calculated relative to the haem content. Molecular weights were calculated (using the residue molecular weights) from the integral values (bracketed figures), (a) assuming 1 haem and 2 cysteines and (b) assuming 2 haems and 4 cysteines. In addition 18 was added for one H_2O The determination assuming 2 haems gives a value closest to the molecular weight determined by SDS-PAGE. Therefore cytochrome c_4 contains 2 haems.

	nmole/ nmole haem		nmole/2 nmoles haem	
ASP	9.65	(10)	19.30	(19)
THR	2.72	(3)	5.44	(5)
SER	4.28	(4)	8.56	(9)
GLU	9.92	(10)	19.84	(20)
PRO	5.62	(6)	11.24	(11)
GLY	13.90	(14)	27.80	(28)
ALA	11.50	(11)	23.00	(23)
VAL	3.90	(4)	7.80	(8)
MET	2.40	(2)	4.80	(5)
ILE	2.40	(2)	4.80	(5)
LEU	7.30	(7)	14.60	(15)
TYR	2.60	(3)	5.20	(5)
PHE	1.97	(2)	3.94	(4)
HIS	1.97	(2)	3.94	(4)
LYS	5.40	(5)	10.80	(11)
ARG	3.10	(3)	6.20	(6)
MW	9812[a]		19379[b]	

MW dehaemed cytochrome c_4 20893

* Note that van Heyningen and Coulson (1987) reported the failure of analysis of amino acid compositions as a probe for sequence similarities in tetanus toxin.

F. Comparison of cytochromes c_4 from Pseudomonas stutzeri 224 with cytochromes c_4 from other bacterial species

The evidence that two proteins are related requires knowledge of either the sequence or the amino acid composition. For the cases where the sequences are known the method of study involves lining up the sequences and, allowing for insertions/deletions, calculating the percentage difference between the sequences. However, for some proteins, including the cytochromes c_4, the sequence data is sparse. Therefore some other method must be used to determine how closely related the proteins are. In these cases the statistical method of Cornish-Bowden (1983) can be used to predict how closely related two proteins are from knowledge of the amino acid compositions. This involves calculating the number of differences between two statistical sequences. One of the basic assumptions of the method is that the probability of finding one type of amino acid at any locus is the same for all loci . From the amino acid analyses the probability of finding a particular amino acid at a particular locus can be calculated for each protein (ie almost like building up a statistical sequence) and then comparing sequences. If the differences between sequences is less than 42% then it can be stated, almost certainly, that the proteins are related. Values of between 42 and 93% gives a weak indication that the proteins are related and values of greater than 93% implies no grounds for believing that the proteins are related.*

Table II shows the amino acid compositions for cytochromes c_4 from Pseudomonas stutzeri 224, Azotobacter vinelandii, Pseudomonas aeruginosa and Alcaligenes sp and the amino acid composition of cytochrome c-552 from Pseudomonas perfectomarinus. Table III shows the results of the Cornish-Bowden analyses.

Pseudomonas stutzeri 224 cytochrome c_4 scores less than 42% against Azotobacter vinelandii and Pseudomonas aeruginosa cytochromes c_4 but scores more than 42 against the other two suggesting a weak indication that they are related. However, with figures of 51 and 47% these are towards the lower end of the weak bracket (42-93). The table also shows that Azotobacter vinelandii is almost certainly related to the other 4 cytochromes. Both the Alcaligenes sp cytochrome c_4 and Pseudomonas perfectomarinus cytochrome c-551 are related to only one of the other cytochromes (Azotobacter vinelandii cytochrome c_4).

Table II : Amino Acid Compositions of Cytochromes c_4 from Pseudomonas stutzeri (stut), Azotobacter vinelandii (vine), Pseudomonas aeruginosa (aerg), Alcaligenes sp. (Alca) and Cytochrome c-552 from Pseudomonas perfectomarinus (perf)

	stut	vine	aerg	Alca	perf
Asp	19	21	18	19	27
Thr	6	9	10	5	14
Ser	9	10	10	6	12
Glu	20	18	15	21	21
Pro	11	11	7	13	11
Gly	28	25	23	18	31
Ala	23	29	28	30	35
Cys	4	4	4	4	3
Val	8	5	6	8	16
Met	5	6	6	5	7
Ile	5	6	9	7	7
Leu	15	14	14	13	15
Tyr	5	6	4	5	9
Phe	4	4	5	3	8
His	4	4	6	3	5
Lys	11	11	11	8	17
Arg	6	7	5	6	7
	183	190	181	174	245

Table III : Comparison of the Amino Acid Analyses by the Method of Cornish-Bowden

The figures are percentage differences between the amino acid sequences.

	stut	vine	aerg	Alca	perf
stut	x	21	38	51	45
vine	21	x	18	32	37
aerg	38	18	x	50	47
Alca	51	32	50	x	62
perf	45	37	47	62	x

CHAPTER IV : INDUCTION AND CELLULAR LOCATION OF CYTOCHROME c_4

Section I - Quantitation of Cytochromes by Purification

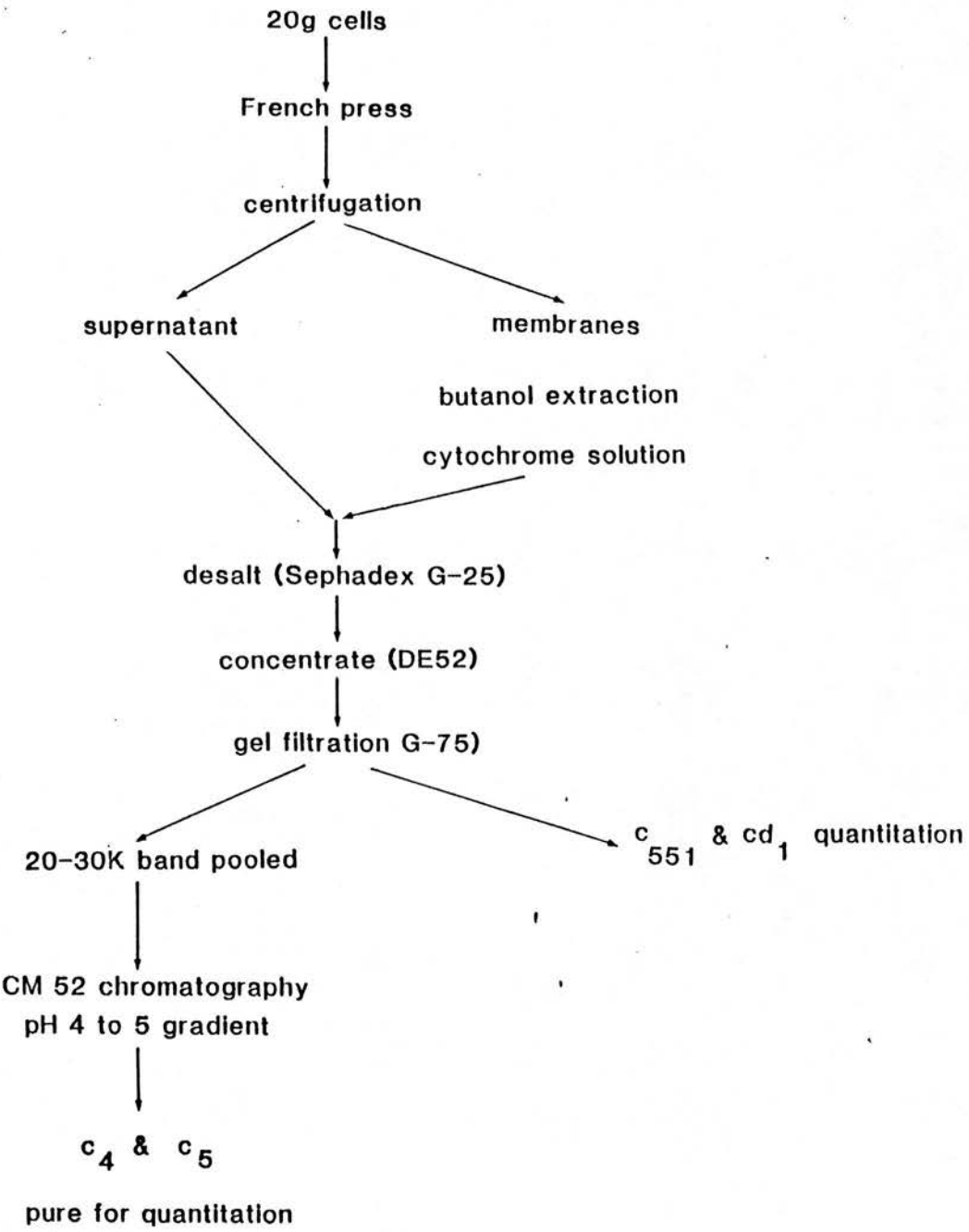
A. Purification method for cytochromes c_4, c_5, cd_1 and c-551.

The purification procedure for Pseudomonas stutzeri 224 cytochromes c_4, c_5, cd_1 and c-551 from both aerobic and nitrate grown cells is briefly outlined in Figure 18. The purification scheme was developed to involve as few steps as possible to obtain the individual cytochromes free from other haem proteins. Contamination from non-haem proteins is permitted since the cytochromes are quantitated spectrally in the 500-580nm range where non-haem proteins do not contribute to the absorbance. A possible exception to this would be the iron-sulphur proteins.

(i) Cell breakage

20g cells were suspended in 80ml 20mM sodium phosphate buffer, pH 7 containing 1mM PMSF and 0.2mg DNase. Cells were ruptured by passing cells through a small aperture at 83 MPa in a French pressure cell. Centrifugation (MSE superspeed 65 with 8x35ml titanium rotor) for 60 mins at 100 000 x g produced a pellet of membranes and a supernatant which contained the soluble cytochromes. Membranes were resuspended in a further 80ml 20mM sodium phosphate buffer, pH 7. Centrifugation for 60 mins at 100 000 x g produced a second supernatant, which was added to the

Figure 18 : Purification Scheme for Cytochromes c_4, c_5, cd_1 and c-551 from Pseudomonas stutzeri 224


20g cells
French press
centrifugation
supernatant
membranes
butanol extraction
cytochrome solution
desalt (Sephadex G-25)
concentrate (DE52)
gel filtration G-75)
20-30K band pooled
c_{551} & cd_1 quantitation
CM 52 chromatography
pH 4 to 5 gradient
c_4 & c_5
pure for quantitation

first, and a membrane pellet which was subsequently suspended in 20ml 20mM sodium phosphate buffer, pH 7.

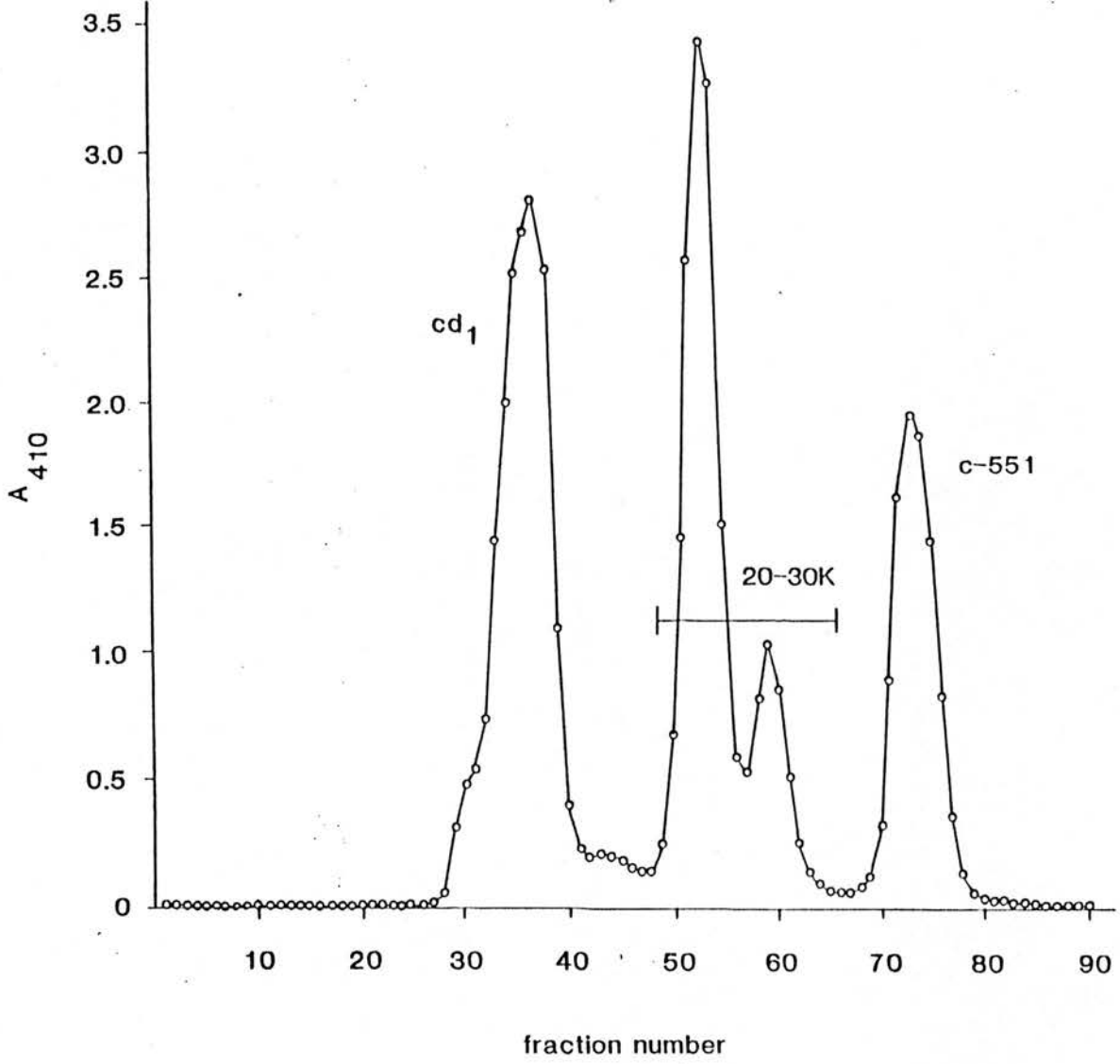
(ii) Purification of cytochromes from the supernatant (soluble cytochromes)

After desalting (Sephadex G-25 coarse) into 5mM Tris-HCl pH 8 (4°C), neutralised sodium ascorbate was added to a final concentration of 1mM to reduce the cytochromes. Reduction was necessary since cytochrome c-551 in the oxidised form absorbs poorly to DE52. This solution was then absorbed onto DE52 (4x5cm) and the column washed through with 100ml 5mM Tris-HCl, pH 8 (4°C). The column was stripped with a solution of 0.2M sodium phosphate buffer, pH 7, typically into a volume of 20-25ml.

The material which was not absorbed onto the DE52 column appeared pink and spectra revealed the presence of both b- and c-type cytochromes, indicative of membranes. These are probably small vesicles, produced at the French press stage, which were not pelleted by the high-speed centrifugation. The pH of this solution was adjusted to pH 5 by the addition of 1M acetic acid. Centrifugation (100 000 x g for 60 mins) produced a membrane pellet (which was added to the main pellet) and a clear supernatant devoid of membranes and soluble cytochromes (determined spectrophotometrically).

The DE-strip was then applied to a column of Sephadex G-75 superfine (3x80cm) equilibrated with 20mM sodium phosphate buffer, pH 7. Figures 19 shows elution profiles from nitrate and aerobic preparations

Figure 19 : Elution of the Nitrate (A) and Aerobic (B) Soluble Extracts from Sephadex G-75


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
A410
cd1
20-30K
c-551
10
20
30
40
50
60
70
80
90
fraction number

B

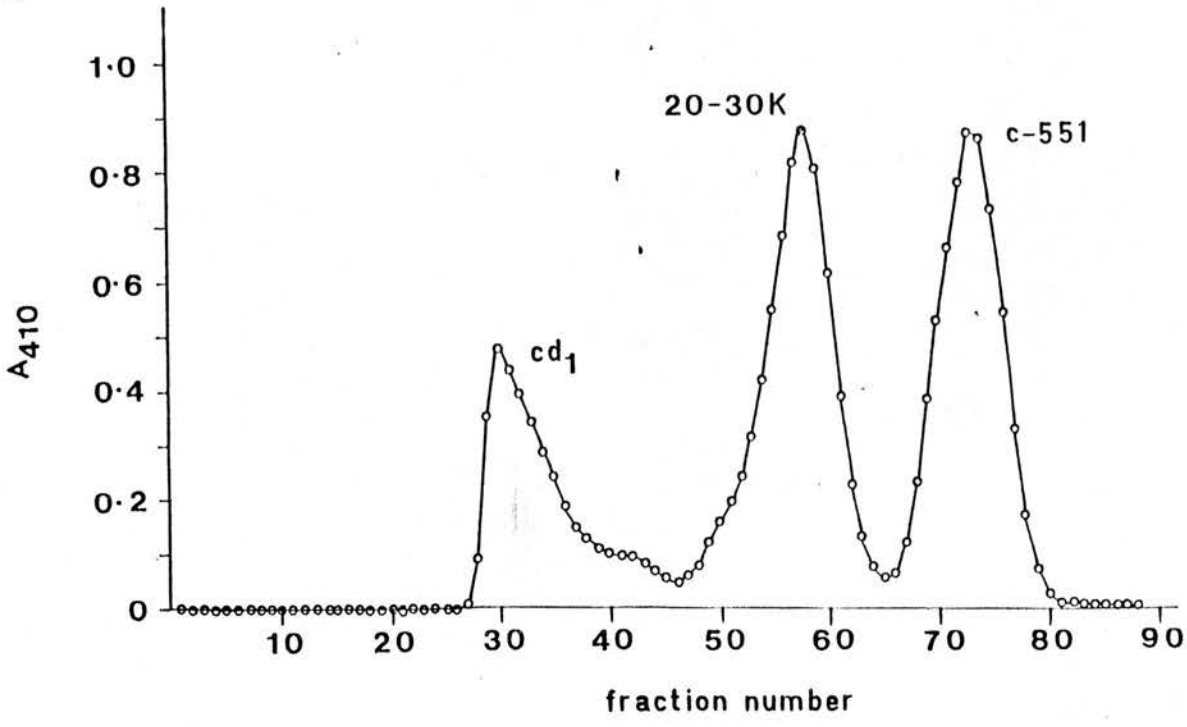
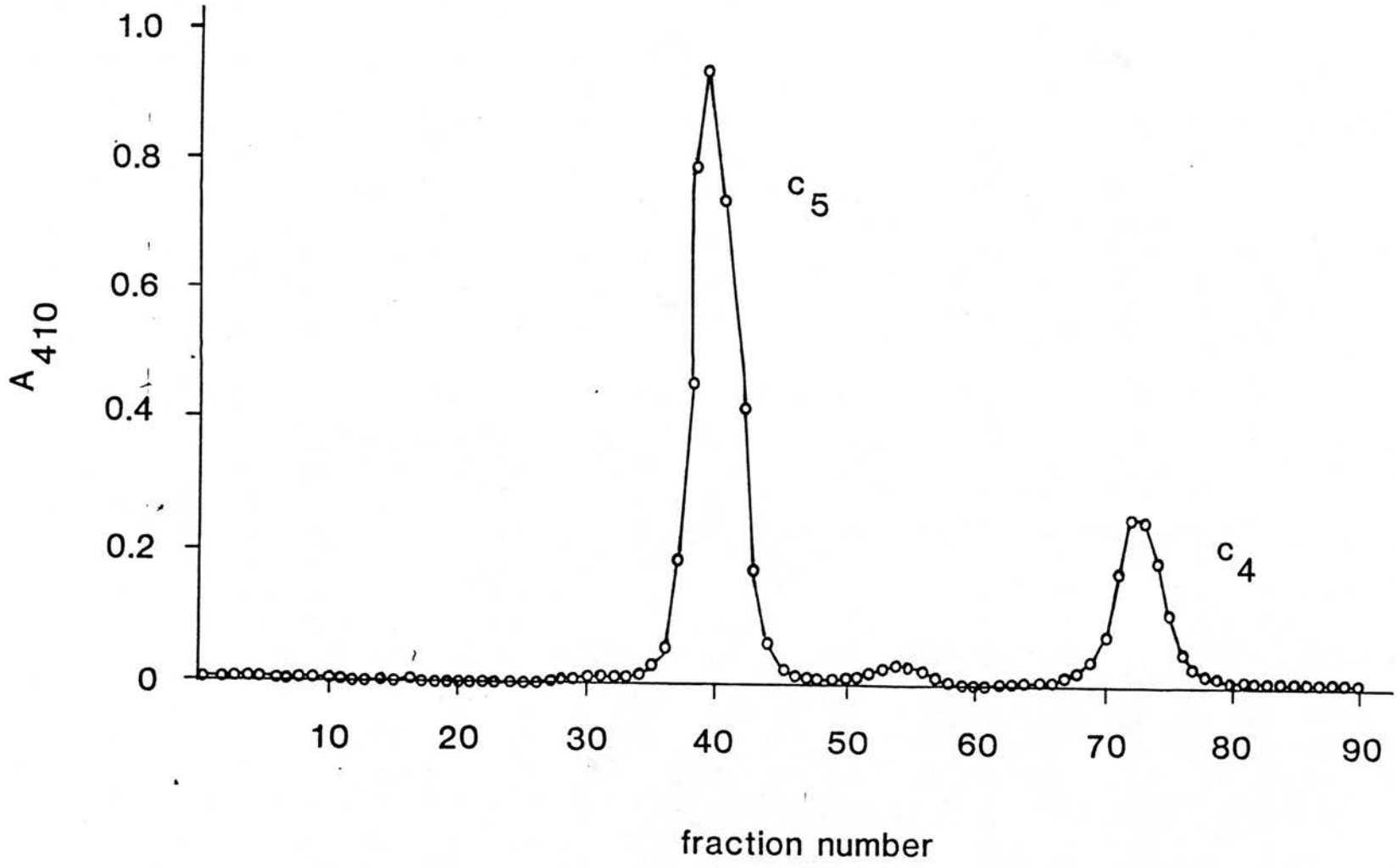

1·0
0·8
0·6
0·4
0·2
0
A410
20-30K
c-551
cd1
10
20
30
40
50
60
70
80
90
fraction number

Figure 20 : Elution of the Pooled 20-30K Fractions of Soluble Cytochromes from Nitrate Grown Cells from CM-52

The elution profile for the aerobic soluble from CM-52 is similar.


1.0
0.8
0.6
0.4
0.2
0
A410
c5
c4
10
20
30
40
50
60
70
80
90
fraction number

respectively. The nitrate preparation shows major peaks for cytochrome cd_1 and a 30K protein (found within the 20-30K region of the profile) which are virtually absent from the aerobic preparation. Figures 23 and 24 lanes d to f show SDS-PAGE analysis of samples taken from each pooled fraction. The evidence from the gels supports the observed difference between the two elution profiles.
The 20-30K pool shows four major cytochromes which include cytochromes c_4 and c_5. It was found necessary to pool this whole peak since, if samples were taken from individual fractions and analysed by SDS-PAGE (not shown), cytochromes c_4 and c_5 were found to some extent over most of the peak. The later fractions of the peaks were found to contain most of the cytochromes c_4 and c_5 whereas the earlier fractions (ie the major peak of the nitrate 20-30K peak) contained mainly 30K.

Since reduced cytochrome c_5 does not absorb well to CM cellulose, sufficient potassium ferricyanide was added to oxidise completely the pooled 20-30K band. The pH of the solution was adjusted to pH 4.0 with 1M acetic acid and it was then absorbed onto CM52 (2x8cm) equilibrated with 10mM ammonium acetate pH 4. The column was then developed with a 50mM ammonium acetate pH 4 to 5 linear gradient (total volume 400ml). Figure 20 shows the elution profile obtained from a nitrate soluble 20-30K application. The elution profile for an aerobic preparation is virtually identical. Only two peaks are eluted from this column which were shown by SDS-PAGE (see Figures 23 and 24 lanes g and h) to be cytochromes c_4 and

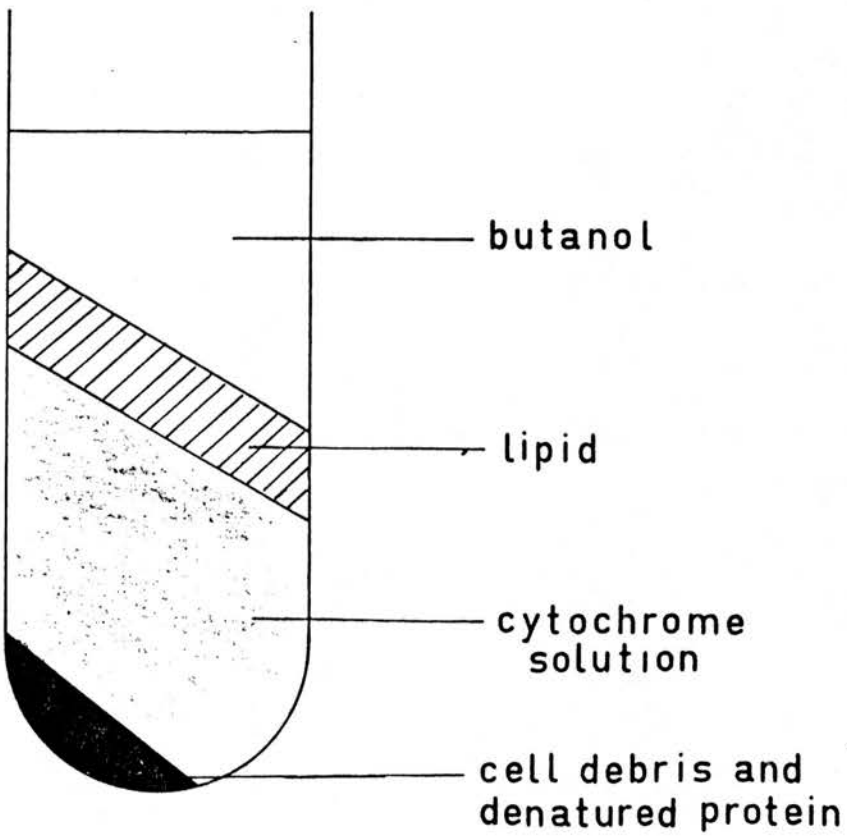
c_5. These cytochromes are now not only free from other haem proteins but also from all other proteins (as determined by staining gels for protein with Coomassie blue). The remainder of the cytochromes in the 20-30K pool (see Figures 23 and 24 lane f) were not eluted from the CM column at this pH range. The cytochrome of around 25000 molecular weight could be eluted using 0.2M sodium phosphate buffer, pH 7 but the 30000 molecular weight cytochrome remained bound at the top of the column.

The pH of the pooled cytochromes c_4 and c_5 fractions were measured, giving values of pH 4.7 and pH 4.4 respectively. The published isoelectric points for Azotobacter vinelandii cytochrome c_4 and cytochrome c_5 are pH 4.69 and pH 4.44 (Swank and Burris, 1969).

(iii) Purification of cytochromes from the membrane fraction

To the suspension of membranes was added an equal volume of pre-cooled (-10°C) butan-1-ol and complete mixing was achieved using a top drive homogeniser. The mixture appeared creamy and pink. This was centrifuged at 14000 x g (Beckman J-21 with JA20 rotor) for 30 mins which produced four layers in the centrifuge tube (see Figure 21). The aqueous phase, which contained solubilised cytochromes, was removed. To the pellet was added 20ml 20mM sodium phosphate buffer, pH 7. The sample was then homogenised and centrifuged as above. The aqueous phase was removed and added to the first batch. The pellet, plus butanol, was discarded. The

Figure 21 : Separation of Butanol Extracted Membranes into 4 Layers Following Centrifugation


butanol
lipid
cytochrome solution
cell debris and denatured protein

aqueous phase was then treated in exactly the same manner as the high-speed supernatant (soluble cytochromes). Figure 22 shows the elution of the nitrate butanol samples from the Sephedex G-75 column.

It has been proposed (Morton, 1950) that butanol acts by removal of membrane lipids from proteins allowing any proteins associated with the membrane, which are sufficiently polar, to be extracted into the aqueous phase; here it is the 20mM sodium phosphate buffer, pH 7 Any integral membrane proteins, or non-polar proteins, are not soluble in the aqueous phase and tend to precipitate and can be removed by centrifugation.

B. Fractionation and Purity of Individual Cytochromes Assessed by SDS-PAGE

Figures 23 to 26 show SDS-PAGE analysis of samples taken at different stages throughout the purification procedure. Although the main purpose of these gels is to follow the purification of cytochromes c_4, c_5, cd_1 and c-551 a little discussion about the fate of the other cytochromes will be included.

(i) Soluble cytochrome preparations

(a) Nitrate soluble: Figure 23 shows a haem stained gel of samples taken at different stages throughout the preparation (stages described in figure legend). The standards (lane a) are purified cytochromes c_4, c_5 and c-551 from Pseudomonas stutzeri 224. Lanes b and c show

Figure 22 : Elution Profiles of Nitrate (A) and Aerobic (B) Butanol Extracts from Sephadex G-75

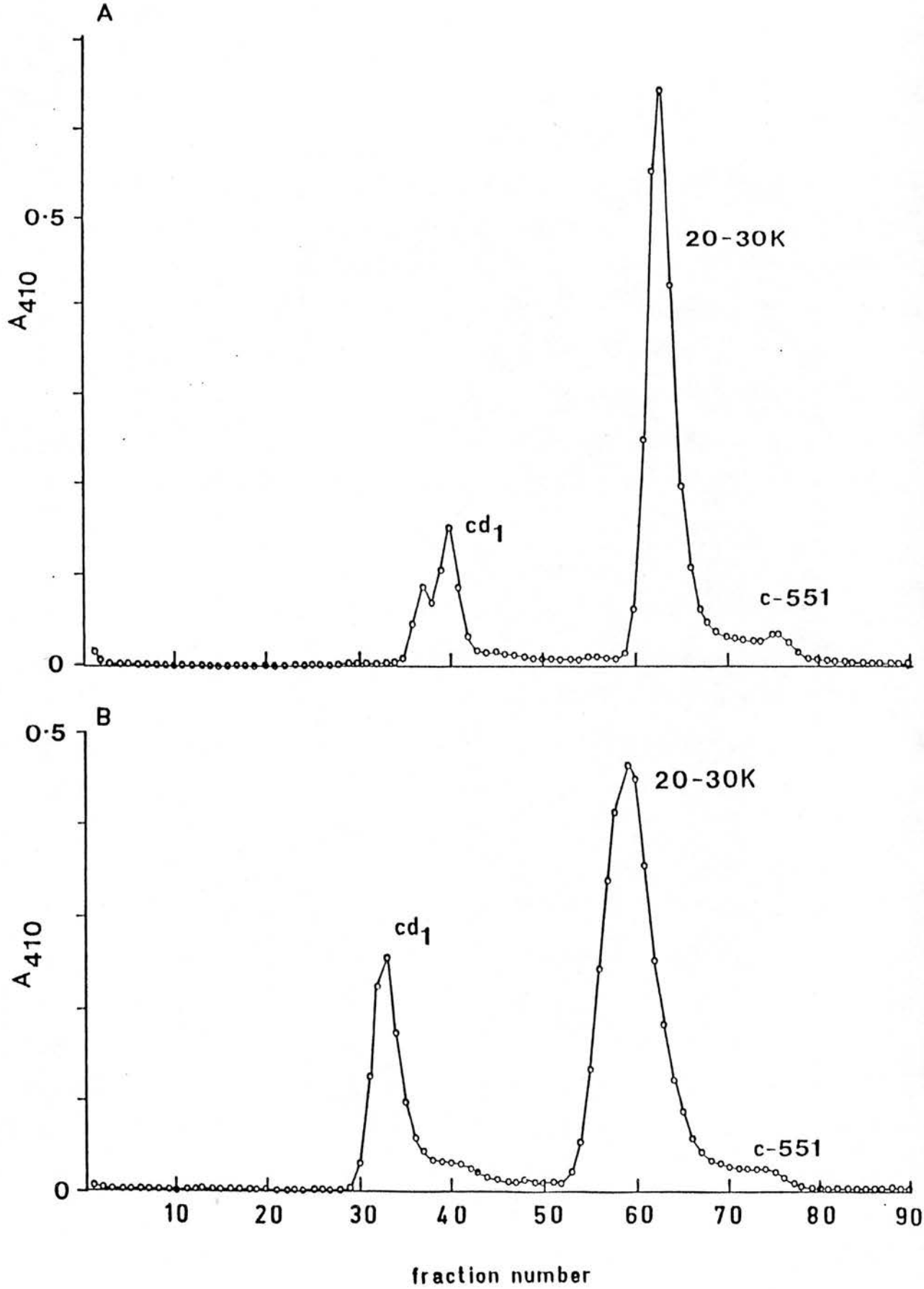
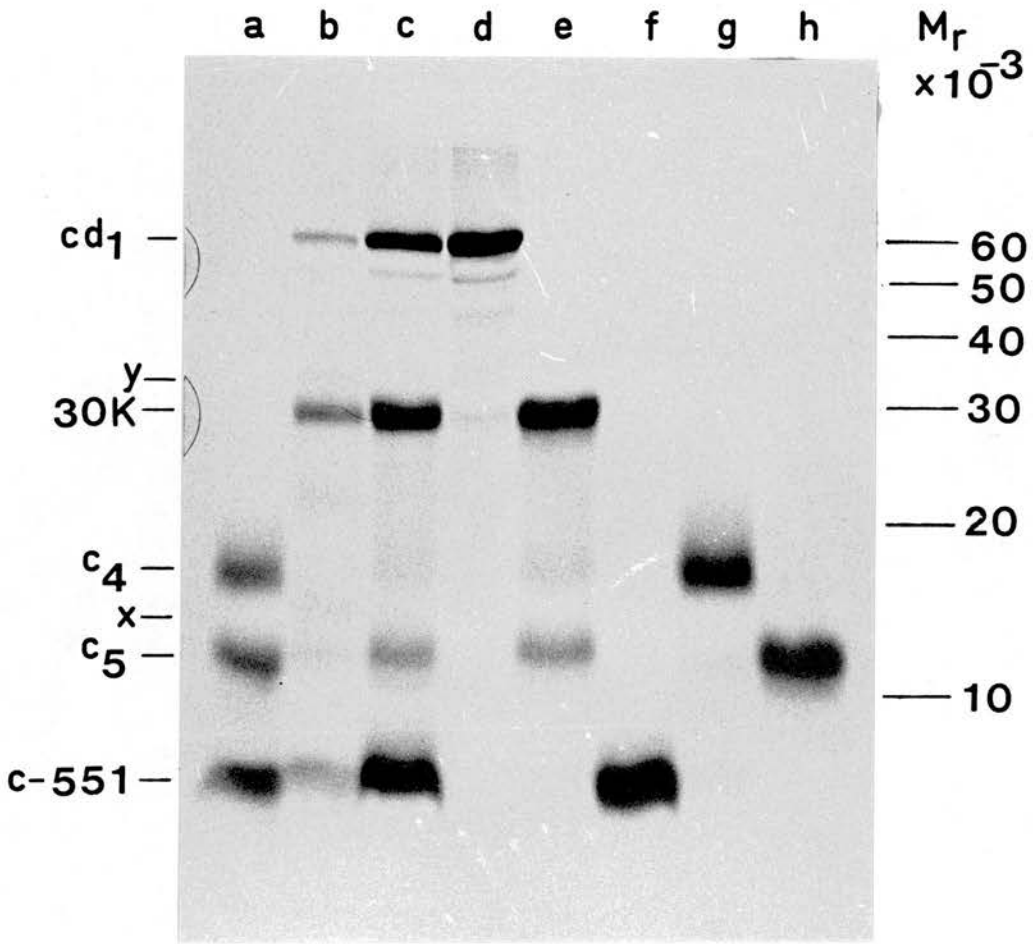

A
0·5
0
A_{410}
20-30K
cd_1
c-551
B
0·5
0
A_{410}
20-30K
cd_1
c-551
10
20
30
40
50
60
70
80
90
fraction number

Figure 23 : SDS-PAGE Analysis of Samples Taken During the Nitrate Soluble Preparation

Lane (a) purified Pseudomonas stutzeri cytochromes c-551, c_4 and c_5. (b) G-25 desalt, (c) DE strip, (d) cd_1 (G-75), (e) 20-30K (G-75), (f) c-551 (G-75) (g) c_4 (CM52) and (h) c_5 (CM52). The M_r scale was calculated from the relative mobilities of a set of molecular weight marker proteins (not shown). Bands marked x and y are present only in lane b and can be shown to be membrane proteins which are not adsorbed onto DE52 (note the absence of bands x and y from lane d).


a
b
c
d
e
f
g
h
Mr
x10-3
cd1
y
30K
c4
x
c5
c-551
60
50
40
30
20
10

the c-type cytochrome complement of unfractionated extracts. Lanes b and c were samples taken to demonstrate any differences in cytochrome complement after absorption to DE52. Only two bands are seen to disappear - ie x and y (marked on diagram), which will be shown in Figure 25 (nitrate butanol preparation), and also in the spheroplast experiments (this chapter section II) to be membrane proteins. Band x will also be shown to be induced under nitrate growth. The presence of membrane proteins before the DE52 (ie in the G-25 desalt) agrees well with the observation that during the purification outline the material not absorbed onto the DE52 column contained membrane proteins. The absence of these membrane proteins from the DE52 strip sample (lane c) was not surprising. The appearance of additional bands in lane c is merely due to these cytochromes being too dilute in the G-25 desalt sample to show up with haem staining, for example cytochrome c_4.

Lanes d to f show samples taken from the pooled peaks from the Sephadex G-75 column (profile shown in Figure 19). Lane d shows that the cytochrome cd_1 is essentially free from other haem proteins. Cytochrome c-551 (Figure 23 lane f) can be seen to be totally free from other haem proteins. It was after the G-75 stage that cytochromes cd_1 and c-551 were quantified.

Lane e shows the 20-30K pool, which contains all the other c-type cytochromes except cytochromes cd_1 and c-551 (of which there are none in the 20-30K pool).

Lanes g and h show samples taken from the two peaks obtained after chromatography on CM52 (see Figure 20 for

the elution profile) and shows them to be pure (at least from other haem proteins) cytochromes c_4 and c_5. The 30K band is not eluted from the CM52 column.

(b) Aerobic soluble: Figure 24 shows samples from the aerobic soluble preparation. For cytochromes c_4, c_5, cd_1, 30K and c-551 the fate is as described for the nitrate soluble preparation. Similarly, band y can be seen in the G-25 desalt sample and absent from the DE-strip. Again band y is a membrane protein (as described in the previous section). The major difference is the amount of cytochrome cd_1 and 30K which are present in the two preparations - ie the quantities are much greater in the nitrate preparation.

For the aerobic soluble preparation additional bands can be seen which run on the gel between the 30K band and the cytochrome c_4. Their fate is the same as that of the 30K band - ie present in the 20-30K pool from the G-75 but not recovered from the CM52 column.

(ii) Butanol (membrane) preparations

(a) Nitrate butanol: Figure 25 shows a gel of samples taken at various stages during a nitrate membrane cytochrome preparation. Lane b is a sample of membranes to demonstrate the complete membrane c-type cytochrome complement.

Figure 24 : SDS-PAGE Analysis of Samples Taken During the Aerobic, Soluble Preparation

Lane (a) is purified Pseudomonas stutzeri cytochromes c-551, c_4 and c_5, (b) G-25 desalt, (c) DE strip, (d) cd_1 (G-75), (e) 20-30K (G-75), (f) c-551 (G-75), (g) c_4 (CM52) and (h) c_5 (CM52). The M_r scale was constructed from the relative mobilities of a set of molecular weight marker proteins (not shown). Band y is only seen in lane b and can be shown to be a membrane protein which is not adsorbed onto DE52 (note absence of y in lane c).

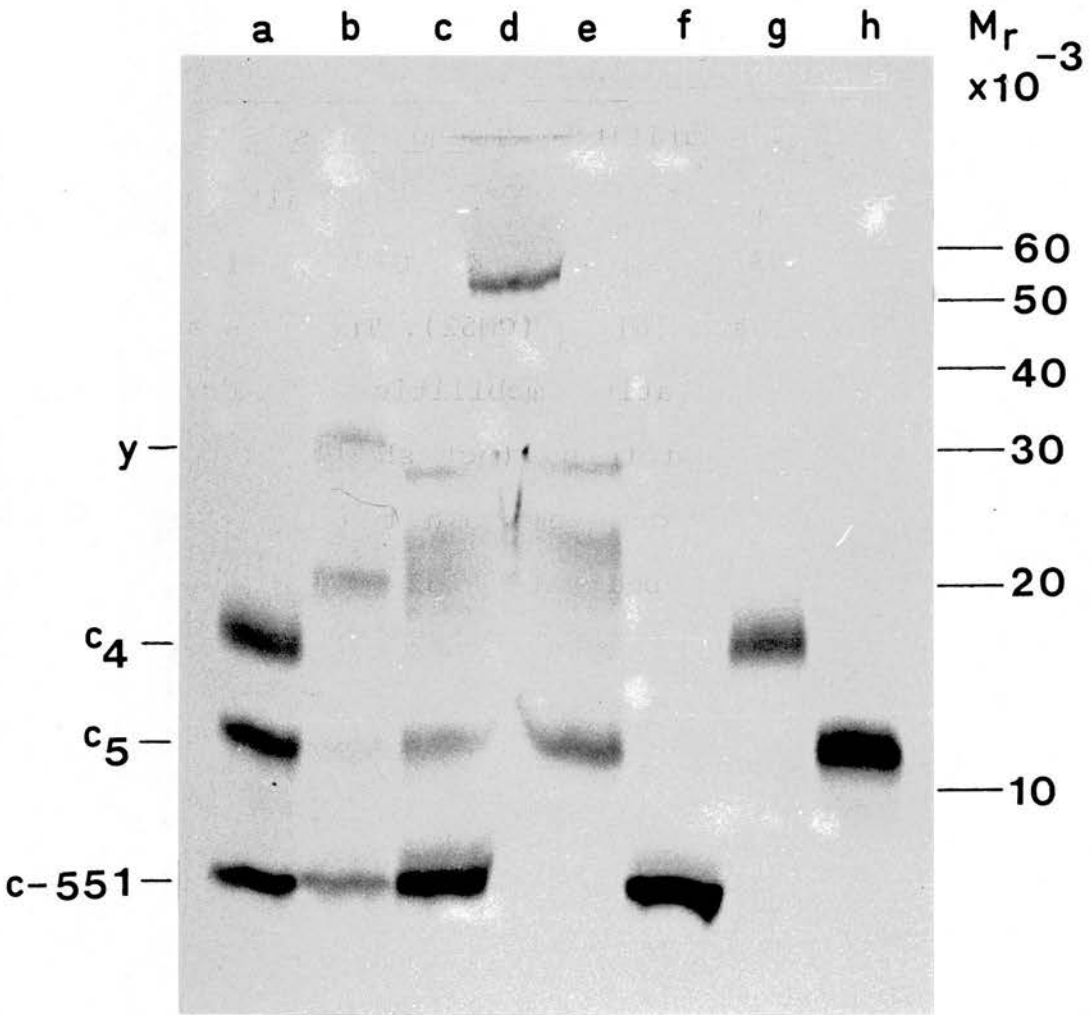
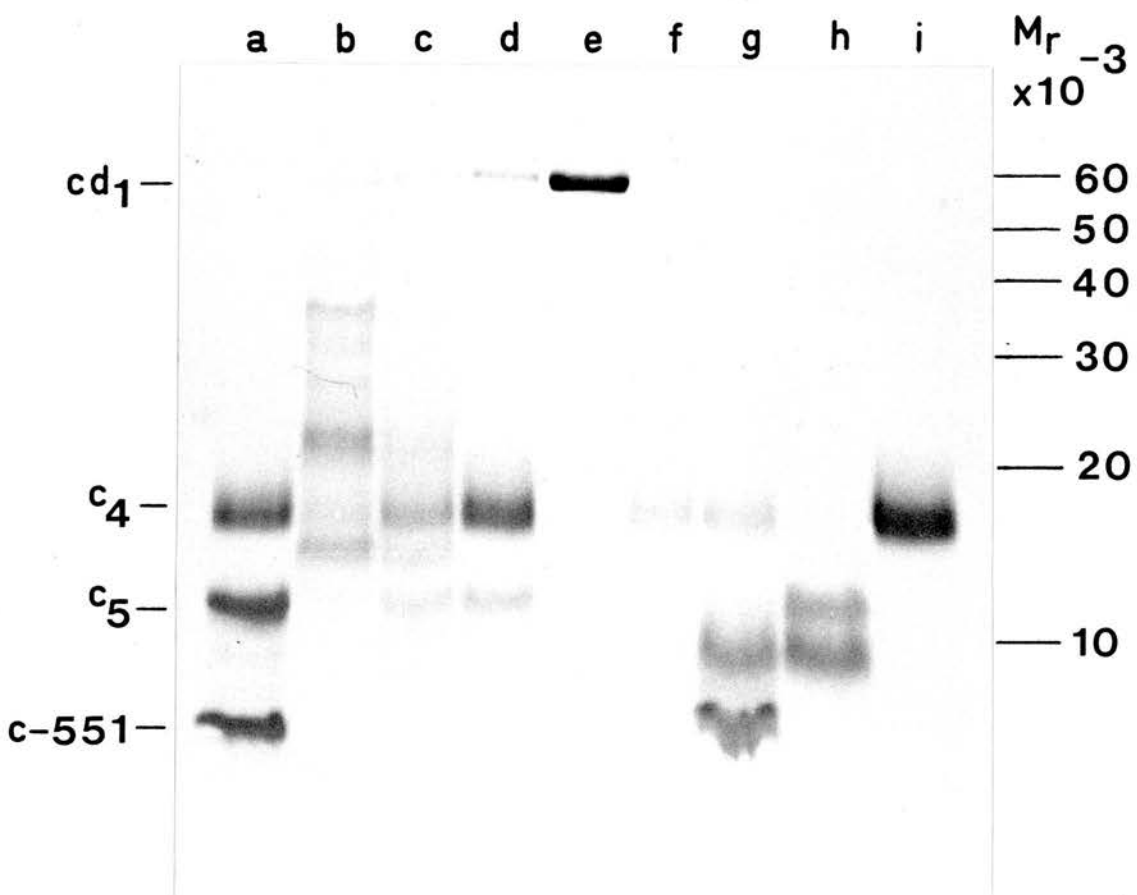

a
b
c
d
e
f
g
h
M_r x10^{-3}
60
50
40
30
20
10
y
c_4
c_5
c-551

Figure 25 : SDS-PAGE Analysis of Samples Taken During the Nitrate, Butanol Preparation

Lane (a) is purified Pseudomonas stutzrei cytochromes c-551, c_4 and c_5 , (b) unfractionated membranes, (c) G-25 desalt, (d) DE strip, (e) cd_1 (G-75), (f) 20-30K (G-75), (g) c-551 (G-75), (h) c_5 (CM52) and (i) c_4 (CM52). The M_r scale was constructed from the relative mobilities of a set of molecular weight marker proteins (not shown).


a
b
c
d
e
f
g
h
i
Mr
x10-3
cd1
c4
c5
c-551
60
50
40
30
20
10

Lane c shows the content of desalted aqueous fraction. It can be seen that in lane c many of the bands (except cytochromes c_4 and c_5) are still present but in lower quantities than in lane b. The intensity of the cytochrome c_4 and c_5 bands is greatly increased in lane c when compared to lane b. If the loadings in lanes b and c were equal with respect to cytochrome c_4 it can be seen that the remainder of the membrane cytochrome bands would become very faint. After concentration on DE52 the only bands remaining are cytochromes c_4, c_5, cd_1 and c-551 see lane d). The remainder of the bands were not absorbed and probably originated from the pellets (ie precipitated protein) at the butanol extraction stage due to incomplete pelleting by centrifugation.

Lanes e, f and g show samples taken from the three main G-75 peaks (see Figure 22 for the elution profile). Lane e shows that cytochrome cd_1 is again essentialy free from other haem proteins. However, the cytochrome c-551 (lane g) is seen to be contaminated with both cytochromes c_4 and c_5. Consideration of the elution profile (Figure 22 explains this contamination. The cytochrome c-551 peak can be seen to be a small shoulder on the main 20-30K peak. Therefore the two elution peaks overlap in some fractions. From quantitation spectra the amount of cytochrome c-551 calculated is 6 nmoles (from approx. 20g starting weight of cells). Densitometer scans reveal that 50% of the cytochrome is cytochrome c-551, 38% is cytochrome c_5 and 12% cytochrome c_4. The amounts of cytochromes c_4 and c_5 are small - ie approx. 2 nmoles cytochrome c_5 and less than 1 nmole cytochrome c_4.

A further purification step does not seem justified because (1) detection of the tiny amounts of cytochrome eluted from a CM52 column would be difficult, if not impossible; and (2) the small amounts of cytochromes c_4 and c_5 are not going to significantly affect the overall distribution diagram.

Lane i shows that after CM52 chromatography of the 20-30K pool the cytochrome c_4 is free from other haem proteins. However, cytochrome c_5 can be seen as two bands - both of which may be cytochrome c_5. Again further purification was not carried out since only a total of 4 nmoles was recovered.

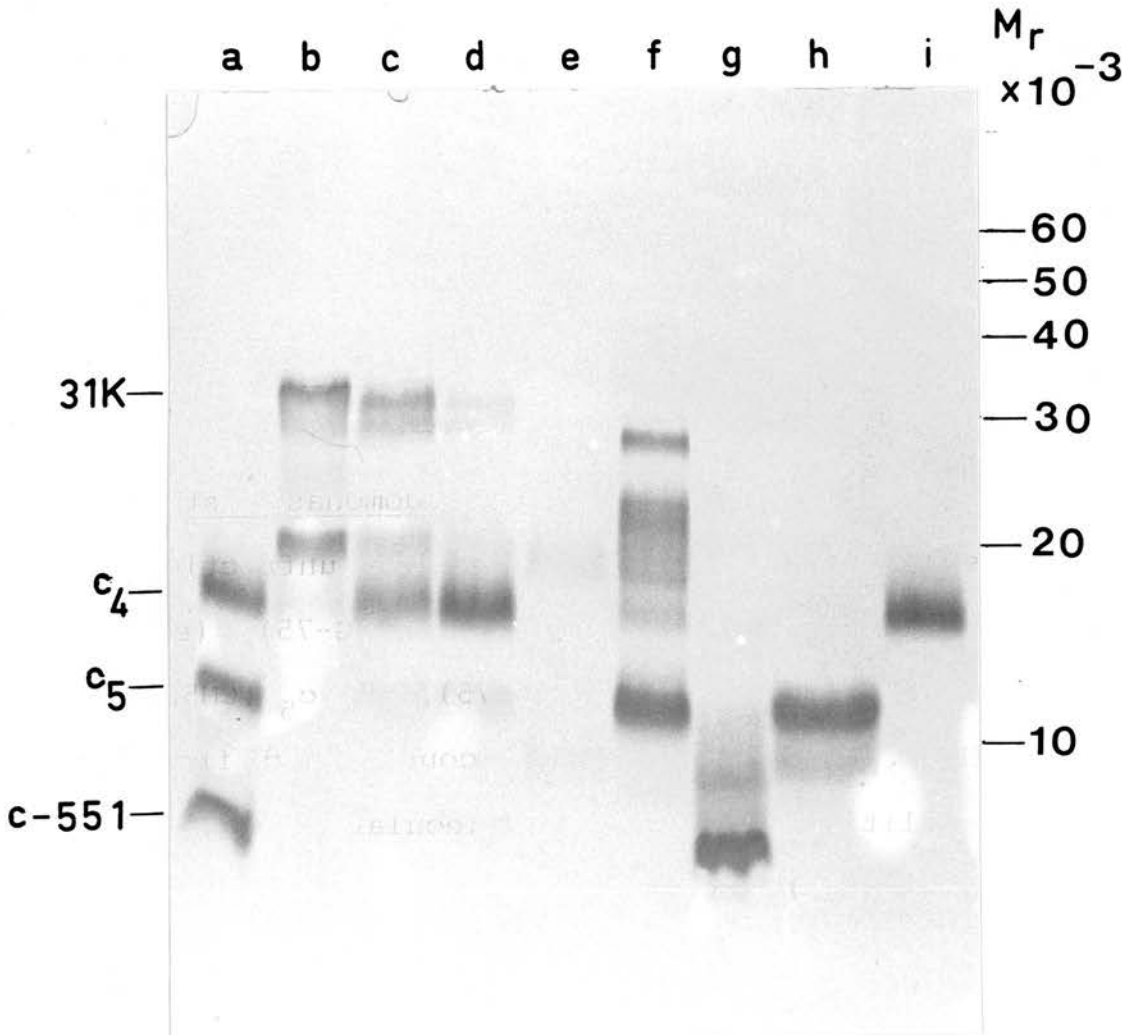
(b) Aerobic butanol : The pattern for purification was exactly the same for cytochromes c_4, c_5 and c-551 as described above. However, no cytochrome cd_1 was observed (see Figure 26 lane e which shows the presence of one of the major membrane bands).

Again for cytochrome c-551 (total recovered was 7 nmoles) cytochromes c_4 and c_5 are contaminants, contributing 6% and 43% to the total "c-551" respectively.

Cytochrome c_5 is present as two bands. It is interesting to note that the proportion of each band is altered depending on the growth conditions. Under aerobic conditions densitometer scans reveal that the heavier band (which runs with the same mobility as the standard (soluble cytochrome c_5)) contributes 78% of the total whereas under nitrate conditions the contribution is reduced to 41%.

Figure 26 : SDS-PAGE Analysis of Samples Taken During the Aerobic, Butanol Preparation

Lane (a) is purified Pseudomonas stutzeri cytochromes c-551, c_4 and c_5, (b) unfractionated membranes, (c) G-25 desalt, (d) 20-30K (G-75), (e) cd_1 (G-75), (f) DE strip, (g) c-551 (G-75), (h) c_5 (CM52) and (i) c_4 (CM52). The M_r scale was constructed from the relative mobilities of a set of molecular weight marker proteins (not shown).


a
b
c
d
e
f
g
h
i
Mr
x10-3
60
50
40
30
20
10
31K
c4
c5
c-551

C. Quantitation of Cytochromes c_4, c_5, cd_1 and c-551 from Aerobic and Nitrate Cells

Individual cytochromes are quantitated from spectra at the α-peak. The α-peak maxima are 550nm for cytochrome c_4, 554nm for cytochrome c_5, 551nm for cytochrome c-551 and 550nm for cytochrome cd_1. The millimolar extinction coefficients used for the calculation were 22.1, 29.3 and 32.9 for cytochromes c_4, c_5 and c-551 respectively (determined as described in methods chapter). Due to cloudiness from membranes the amount of cytochrome cd_1 was estimated from reduced minus oxidised spectra using 20 as millimolar extinction coefficient.

The results of a quantitation experiment are shown diagramatically in Figure 27 and are expressed in nmoles/100g cells.

(i) Cellular location of the individual cytochromes

The diagram shows clearly that cytochromes c_5, cd_1 and c-551 are found predominantly in the soluble fraction and that cytochrome c_4 is found both in the membrane and soluble fraction, but the largest proportion is found in the membrane.

The purification of cytochrome c-551 from membranes may be due to entrapment of soluble proteins within French press membrane vesicles. The amount of cytochrome c-551 isolated from membranes represents less than 2% of the soluble cytochrome c-551.

(ii) Induction of individual cytochromes

Figure 27: Block Diagram Showing the Distribution of Cytochromes c_4, c_5, cd_1 and c-551 Between Membrane and Soluble Fractions from Both Aerobic and Nitrate Grown Cells

Bars represent nmol/100g cells.

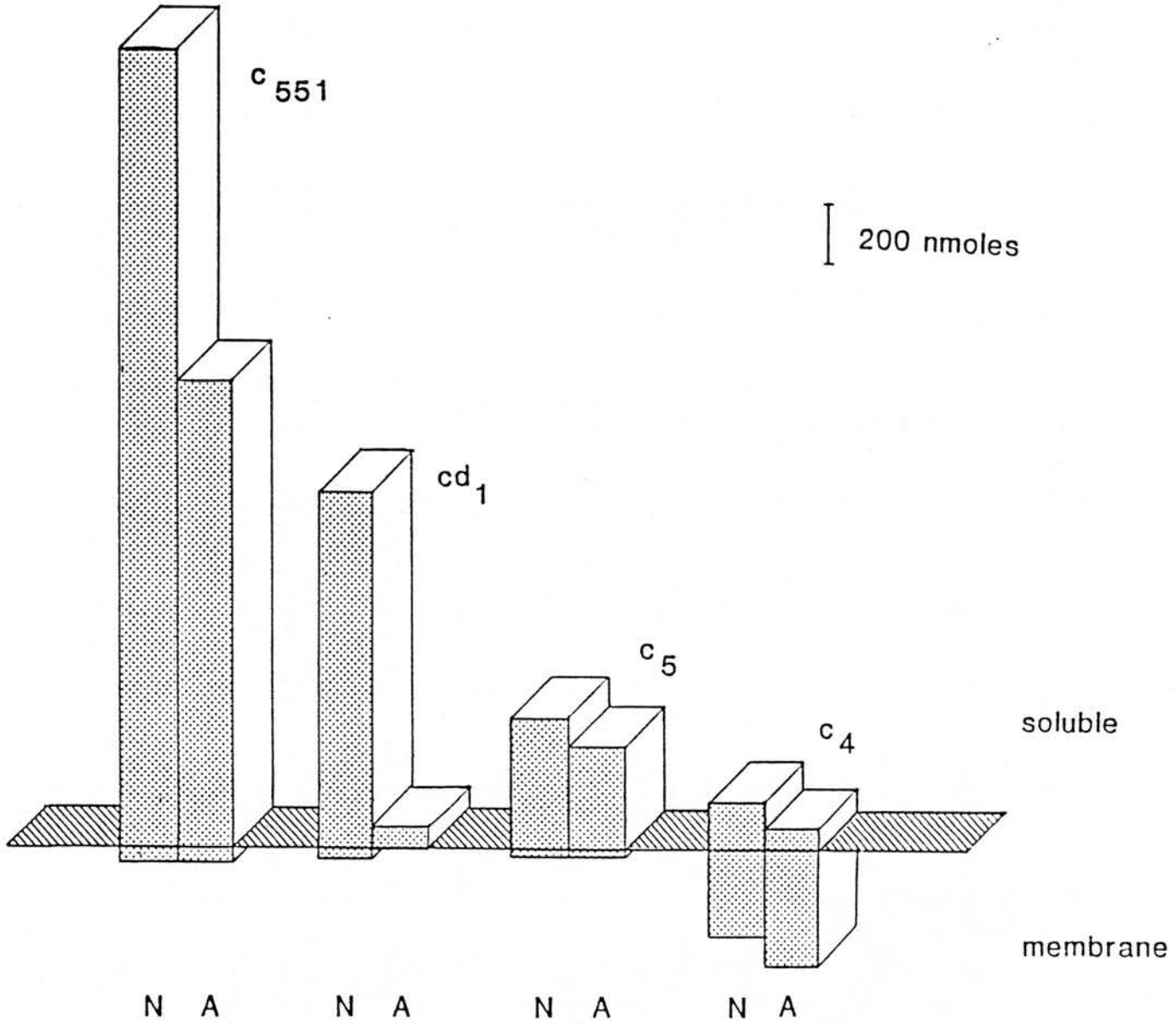

c 551
200 nmoles
cd 1
c 5
c 4
soluble
membrane
N A
N A
N A
N A

Figure 27 shows that cytochrome cd_1 is greatly induced under nitrate conditions (16.3 fold increase). This is hardly surprising since cytochrome cd_1 is the nitrite reductase of Pseudomonas (Barber et al, 1976). Cytochrome c-551 is also induced, 1.74 fold increase, under nitrate growth. This is consistent with the result of Parr et al (1976) who demonstrated increased levels of cytochrome c-551 in nitrate grown Pseudomonas aeruginosa. The fact that there are large amounts of cytochrome c-551 under both aerobic and nitrate conditions would tend to indicate that cytochrome c-551 plays no unique role in nitrate respiration (denitrification). The rise may reflect the change from a membrane bound oxidase (possibly cytochrome o) to the soluble terminal acceptor cytochrome cd_1. Cytochrome c-551 has been shown to donate electrons to cytochrome cd_1 of Pseudomonas aeruginosa (Horio, 1961, Barber et al, 1976).

It has been proposed (Reigler et al, 1984) that electron transfer from cytochrome c to photosynthetic reaction centres occurs via two dimensional diffusion of cytochrome c parallel to the membrane surface - ie cytochrome c would be reduced by the reductase and then diffuse across the membrane surface to the electron acceptor. An equivalent system may operate between cytochrome c-551 reductase and cytochrome c-551 oxidase in Pseudomonas stutzeri. However, if the terminal acceptor is not membrane bound but soluble, as is cytochrome cd_1, a three dimensional diffusion would be required through the aqueous phase of the periplasm. It

would therefore seem reasonable to assume that more cytochrome c-551 would be required to maintain the rate of electron transfer.

The level of cytochrome c_5 is seen to be slightly elevated in nitrate grown cells. The significance of this is unknown, as is the function of cytochrome c_5. It is not known if cytochrome c_5 is a unique cytochrome with a specific function or if it is a proteolytic fragment of a larger protein. It is known from N-terminal analysis of cytochrome c_5 from Pseudomonas mendocina that it has a ragged N terminus (Ambler and Taylor, 1973) which may support the idea that cytochrome c_5 is a proteolytic cleavage product of a larger protein.

The case for cytochrome c_4 is more complex. If the total amount of cytochrome c_4 is considered then there is no difference between the amounts found in aerobic and nitrate cells, suggesting no unique role for cytochrome c_4 in denitrification. However, Figure 27 shows that there is a difference in cytochrome c_4 distribution between the membrane and soluble fractions. 85.2% of the cytochrome c_4 is membrane bound in aerobic cells. This is reduced to 65.6% in nitrate grown cells. A possible explanation for this will be put forward in the discussion chapter.

Since it is unusual to find a protein which is both membrane bound and soluble, it is important to demonstrate that both are in fact identical. This is discussed in this Chapter Section IV.

CHAPTER IV : INDUCTION AND CELULLAR LOCATION OF CYTOCHROME C_4

Section II - Quantitation of Individual Cytochromes from Haem Stained Gels

The main advantage of quantifying cytochromes by purification is that it is absolutely certain that the cytochrome is the one of interest. This can be checked by many protein chemical criteria - eg amino acid analysis, spectra and N-terminal analysis. However purification does have disadvantages. During each purification stage losses of protein will inevitably occur. These losses may be of two types: (1) equal loss of all proteins during the purification or (2) preferential loss of one of the components. The purification scheme was designed so that each purification was the same and therefore direct comparisons could be made. No attempt was made to examine losses of individual cytochromes since this would involve adding a labelled cytochrome and follow its recovery at each stage of the purification. For example, cytochromes c_4, c_5 and c-551 could be labelled with different isotopes allowing analysis of losses of each.

Purification for the purpose of quantitation is a time consuming process and because of this the results in Figure 27 represent those of one experiment. To confirm these results either another purification experiment or some other method of quantitation is required. To this end analysis of cell extracts by SDS-PAGE stained for

haem were used. The major advantages of this method are speed and no losses of cytochrome since no purification steps are involved. Cells are grown overnight and then treated to produce soluble and membrane extracts by spheroplast production (see Section A part (ii)). Samples from each cell fraction are applied straight into the gels, stained for haem and then densitometer scans are recorded. Cells, both aerobic and nitrate, can be analysed simultaneously and the whole experiment can be completed in one day. By purification approx. 4-5 weeks would be required.

A. Characterisation of the haem staining method

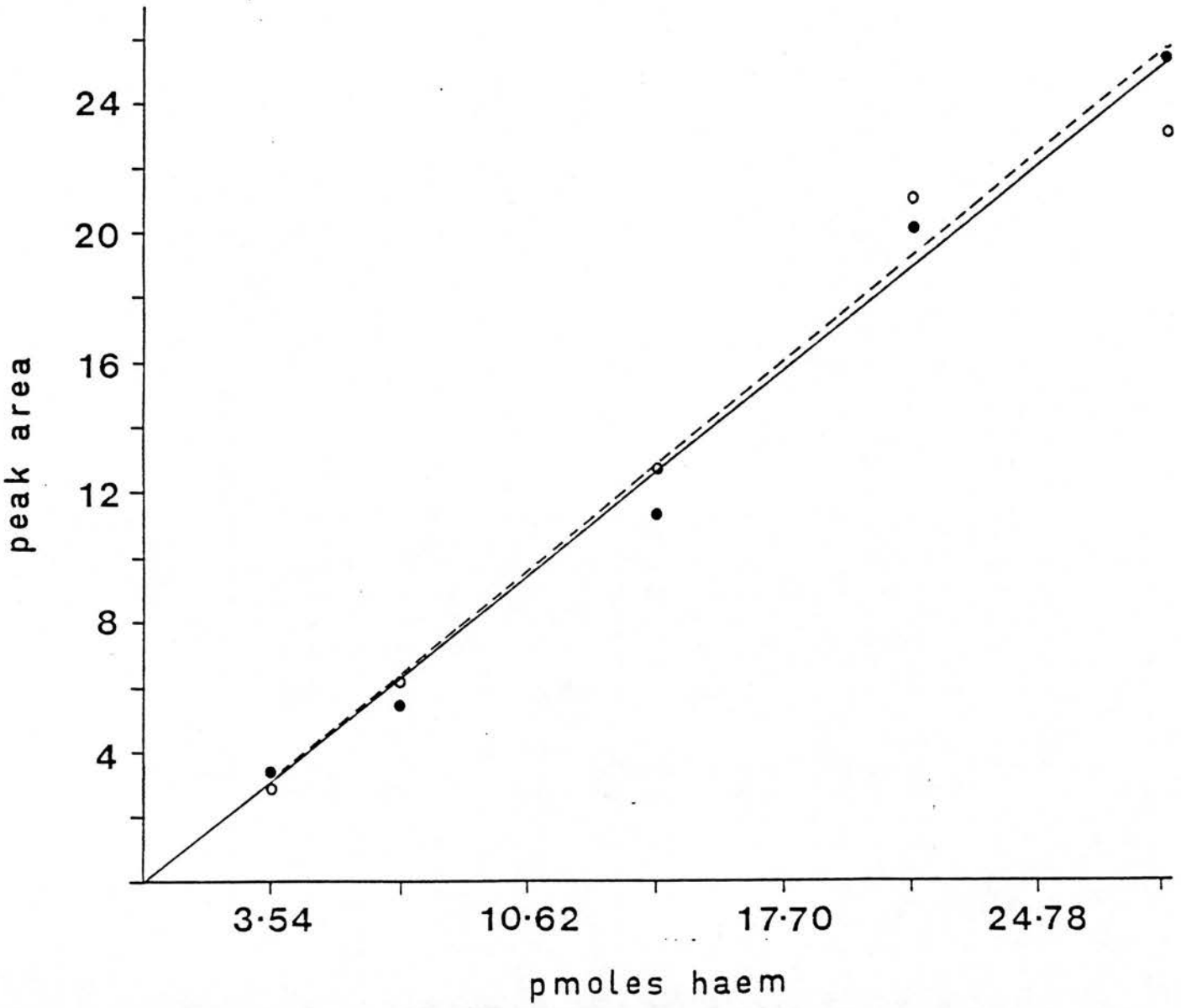
The characterisation of the method has been reported in detail by Goodhew et al (1986), see Appendix I. In addition to all the precautions mentioned by Goodhew et al (1986) all samples were pre-oxidised by addition of potassium ferricyanide prior to addition of SDS, since Fe II dissociates from the haem in SDS yielding a porphyrin which is invisible by haem staining. The Fe III does not dissociate and therefore preoxidation allows all the c-type cytochromes to contribute to the haem staining reaction (refer to methods/proprties).

(i) Linearity of the intensity of colour yield against increased haem loading

Figure 28 shows a curve of increased loading of cytochrome c_4 against area under densitometer scanned gel peaks. For this experiment full oxidation of the cytochrome is ensured by addition of ferricyanide to a

Figure 28 : Curve Showing peak Area Against pmoles Haem Loaded

Peak area is calculated by scanning a haem stained gel. The area is expressed in arbitary units. The plot shows the results from 2 gels.


peak area
24
20
16
12
8
4
3·54
10·62
17·70
24·78
pmoles haem

final concentration of 1.25mM. Both curves show that the response is linear over the range studied.

In all subsequent gels a standard quantity of haem will be run since, even with standardised conditions for staining, there is some inconsistency in colour yields between gels.

(ii) Preparation of spheroplasts

The method detailed below is essentially the same as the method of Garrard (1971) and Wood (1978) except cells were suspended to 0.1g/ml.

Cells were harvested by centrifugation and washed free from remaining growth medium by resuspension in approx. 10 volumes of 10mM sodium phosphate buffer, pH 7, followed by centrifugation. The cells were suspended to 500mg/ml in 10mM sodium phosphate buffer, pH7. At this stage it is important not to store the cells, even for a relatively short time, in Tris-HCl since this causes lysis of the cells. 2ml of cells was then added to a mixture containing 0.5M sucrose, 40mM Tris-HCl pH 8 ($4^{o}C$), 0.25mg/ml lysozyme, 4mM EDTA and trace DNase. This mixture was allowed to stand for 2 mins at room temperature after which magnesium chloride was added to a final concentration of 10mM, giving a final volume of 10ml and cell concentration of 0.1g/ml. This was incubated for 30 mins at $30^{o}C$. Trace DNase was present during the $30^{o}C$ incubation because, although the spheroplasts remain approx. 98% intact, the small amount of DNA released causes the medium to become very viscous thus hindering efficient pelleting of the spheroplasts.

The mixture was centrifuged, 15000 x g for 30 mins (4°C) (Beckman J21 with JA20 rotor). The supernatant contained all the periplasmic proteins while the pellet was spheroplasts (membrane vesicles containing the cytoplasmic contents). Lysis of the spheroplasts (release of cytoplasmic contents) was achieved by resuspending the pellet in 10mM Tris-HCl pH 8 (4°C), 2mM EDTA and trace DNase. This was allowed to stand at room temperature for 30 mins. Magnesium chloride was added to a final concentration of 4mM, giving a final volume of 10ml. This was centrifuged as above. The supernatant contained the cytoplasmic contents and the pellet was membranes which were resuspended to a total volume of 10ml in 10mM Tris-HCl pH 8 (4°C). Figure 29 summarises the spheroplast procedure and shows the production of three cell fractions (ie periplasm, cytoplasm and membranes). Samples taken from each of the three fractions were analysed by SDS-PAGE stained for haem. Densitometer scans and areas under peaks were recorded.

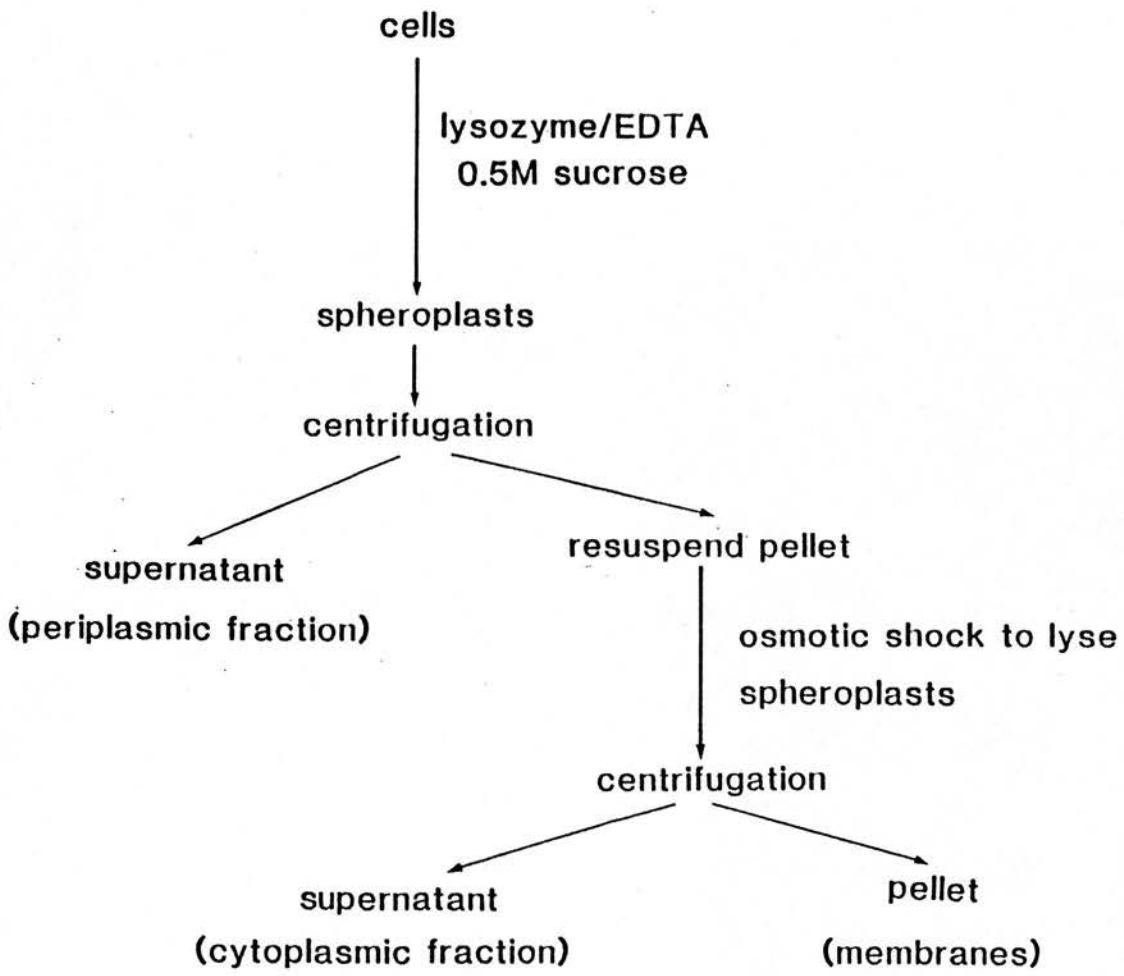
B. Cellular location and quantitation of individual cytochromes by SDS-PAGE stained for haem

(i) Cellular location

One of the major advantages of the spheroplast method is that the celullar location of the soluble cytochromes can be pinpointed more accurately. French pressing cells produces two fractions, membrane and soluble, the soluble fraction being composed of both periplasmic and cytoplasmic proteins. As Figure 29

Figure 29 : Preparation of Spheroplasts

Scheme shows the preparation of 3 cell extracts; periplasm, cytoplasm and membranes. The intactness of spheroplasts is assessed after the first centrifugation by measuring the ICDH activity of the periplasm and lysed spheroplasts (described in detail in text).


cells
lysozyme/EDTA
0.5M sucrose
spheroplasts
centrifugation
supernatant
(periplasmic fraction)
resuspend pellet
osmotic shock to lyse
spheroplasts
centrifugation
supernatant
(cytoplasmic fraction)
pellet
(membranes)

shows, spheroplasting produced three fractions, namely a membrane fraction and two soluble fractions (periplasmic and cytoplasmic). Now the soluble cytochromes can be assigned to either periplasm or cytoplasm.

Figure 30 shows a gel of a spheroplast experiment with periplasmic, cytoplasmic and membrane fractions from both aerobic and nitrate cells. The first important point to note from the gel is that the cytoplasmic fractions are virtually devoid of c-type cytochromes. The cytochromes seen in the cytoplasm can be matched to both periplasmic and membrane cytochromes. The membranes may be explained by incomplete pelleting of the membrane fraction. The periplasmic protein contamination is probably due to incomplete release at the periplasmic stage.

The extent of the lysis of the spheroplasts at the periplasmic stage was assessed by measuring the release of the cytoplasmic enzyme, isocitrate dehydrogenase, into the periplasmic fraction. Table IV shows the results of such an assay demonstrating that small amounts of lysis occurred.

The results here, showing that c-type cytochromes are membrane bound or are located in the periplasm, are in agreement with the hypothesis of Wood (1978) who proposed that c-type cytochromes are located in the cell periplasm or attached to the periplasmic side of the membrane.

The gel also shows that cytochromes c-551 and cd_1 are exclusively periplasmic proteins. The gel also

TABLE IV : ICDH Activities of Cytoplasmic, Periplasmic and Membrane Fractions for Determination of Intactness of Spheroplasts

	Aerobic cells	nitrate cells
Cytoplasmm	96.1	98.3
Periplasm	3.7	1.5
Membrane	0.2	0.2

Figures expressed as percentage of the total ICDH activity.

indicates that cytochrome c_5 is a periplasmic protein and that cytochrome c_4 is distributed between membrane and periplasmic fractions, with most being membrane bound. Thus, the distribution pattern of cytochromes c_4, c_5, cd_1 and c-551 between periplasm (soluble) and membrane fractions confirms the distribution found in the purification and quantitation experiment.

Since no fractionation procedures have been used the samples on the gel show the whole complement of c-type cytochromes and therefore location of the other bands can be examined. For example, there is a band of approx. 30K molecular weight which is located in the periplasmic fraction, some properties of this protein are reported in Chapter VII. Also a c-type cytochrome of approx. 31K is located solely in the membrane. This 31K band may be the equivalent of mitochondrial cytochrome c_1.

(ii) Quantitation and induction of c-type cytochromes

Qualitatively the gel (Figure 30) shows massive induction of cytochrome cd_1 and the 30K band when cells are grown on nitrate. There is also an increase in the amount of cytochromes c-551 and c_5 on nitrate growth. The distribution pattern of cytochrome c_4 is also the same as the purification experiment, namely more soluble cytochrome c_4 in nitrate grown cells with the overall levels the same under both growth conditions.

Quantitation of individual cytochromes was achieved by scanning the gel and Figure 31 shows the resulting densitometer scans produced from the gel in Figure 30. The standard lane (lane a) contains a known loading of

Figure 30 : SDS-PAGE Analysis of Periplasmic, Cytoplasmic and Membrane Fractions from Aerobic and Nitrate Grown Cells Stained for Haem

Lane (a) standard loading of cytochrome c_4 (71pmoles haem), (b) aerobic membranes, (c) 0.4 loading aerobic membranes, (d) aerobic periplasm, (e) 0.4 loading aerobic periplasm, (f) nitrate membranes, (g) 0.4 loading nitrate membranes, (h) nitrate periplasm, (i) 0.4 loading nitrate periplasm, (j) aerobic cytoplasm and (k) nitrate cytoplasm. The M_r scale was constructed from the relative mobilities of a set of molecular weight standards (not shown).

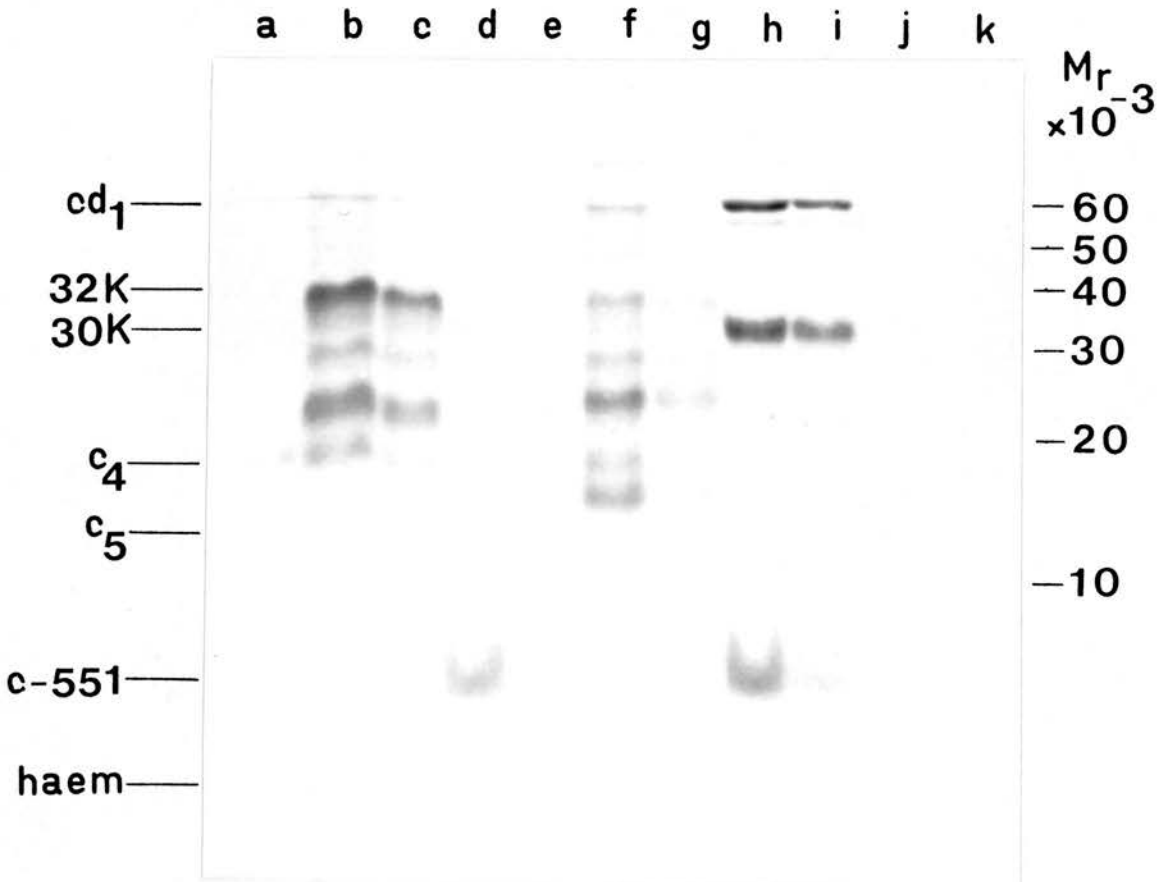
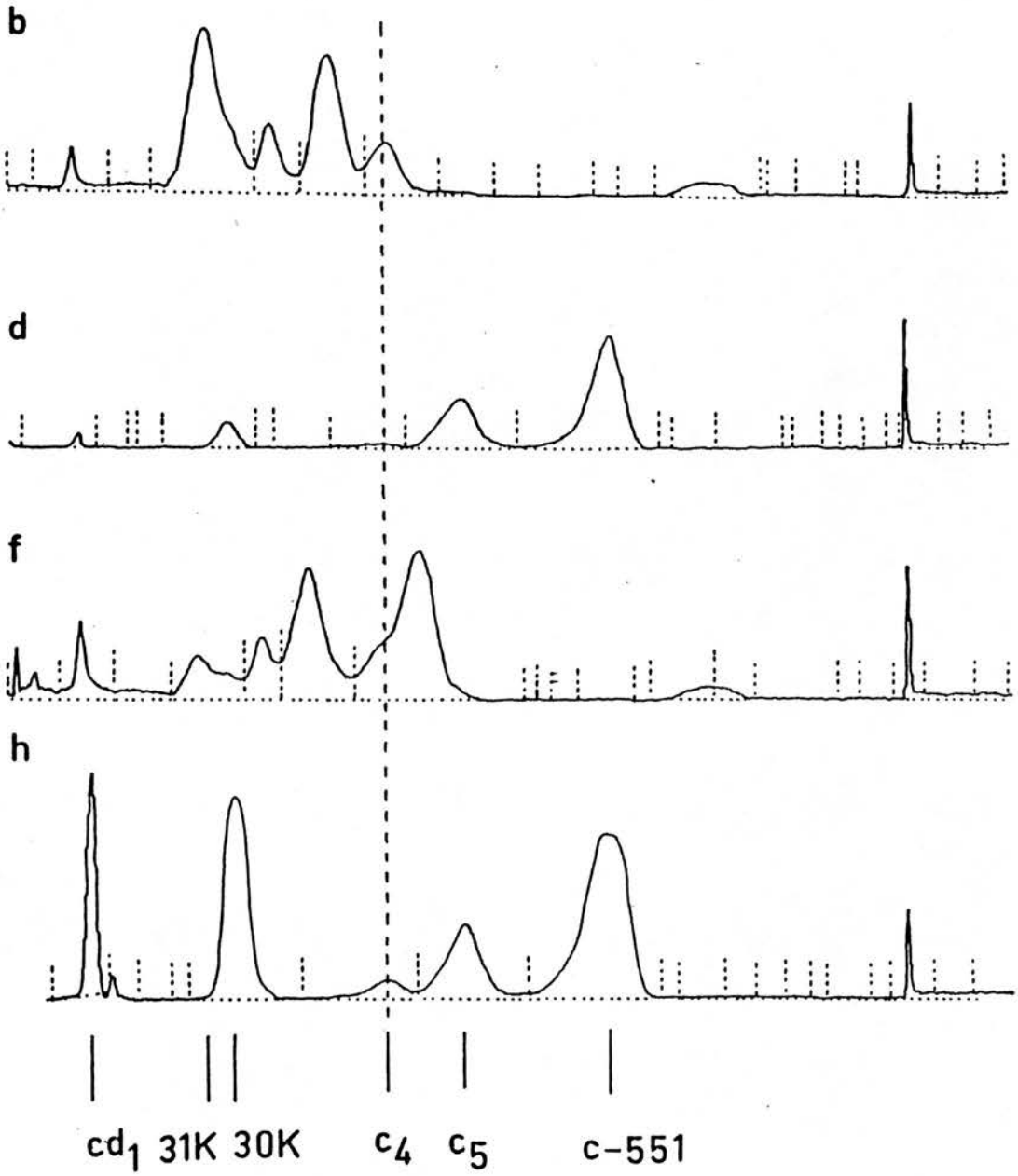

a
b
c
d
e
f
g
h
i
j
k
Mr
×10-3
cd1
32K
30K
c4
c5
c-551
haem
60
50
40
30
20
10

Figure 31 : Densitometer Scans of Selected Lanes from the SDS-PAGE Analysis Shown in Figure 30

Labelling of scans corresponds to the lane labelling in Figure 30. ie (b) aerobic membranes, (d) aerobic periplasm, (f) nitrate membranes and (h) nitrate periplasm.


b
d
f
h
cd1
31K
30K
c4
c5
c-551

cytochrome c_4 (haem) to assess the colour yield of the haem stain. This standard was run with each gel since the colour yield varies between gels even with the standard times for staining. Since the colour intensity has been shown to increase linearly with increasing haem loading (up to a certain point) the amount of haem under each peak can be calculated proportionally from the standard loading. And simply by multiplication the amount of haem under the peak in the sample can be expressed as nmol/100g cells. The results for two separate experiments are shown in Table V. The values from the purification experiment are included for comparison.

With three exceptions the values obtained by purification are lower than the gel method which reflects the losses during the purification procedure. The most notable exception is the quantitation of the aerobic soluble cytochrome c_4. This is probably due to the sensitivity of the haem stain method. Since such small amounts of cytochrome c_4 are found in the sample, either haem staining itself is unreliable at these low levels of loaded haem or the gel scanner cannot detect the peak efficiently enough. Attempts have been made to concentrate samples up to 5 fold (by freeze drying) but this is unreliable since the amount of haem staining did not show a 5 fold increase in intensity. This may be due to incomplete dissolution of the freeze dry pellet. The cytochrome c_5 peak was used for this assessment since a 5 fold increase would not take the amount of staining into

Table V : Quantitation of Cytochromes from Haem Stained Gels

The cytochromes were quantitated from gels where samples were not concentrated before application to the gel (see text). The method for quantitation is also described in the text. The results from the purification experiments and the two gel quantitations were carried out on different batches of cells.

* not detectable on gel

AS aerobic soluble

AM aerobic membrane

NS nitrate soluble

NM nitrate membrane

	Purification (Fig 27)	gel (Fig 30)
	nmol/100g cells	
AS c_4	71	63
AS c_5	366	723
AS c-551	1610	1562
AS cd_1	85	47
AM c_4	410	634
AM c_5	22	*
AM c-551	35	*
AM cd_1	0	*
NS c_4	161	282
NS c_5	464	1085
NS c-551	2796	2977
NS cd_1	1383	767
NM c_4	309	-
NM c_5	21	0
NM c-551	28	0
NM cd_1	31	*
Ratio $\frac{\text{NS c-551}}{\text{AS c-551}}$	1.74	1.91
Ratio $\frac{\text{NS } cd_1}{\text{AS } cd_1}$	16.3	16.3

the non-linear part of the staining curve.

The other main exception is cytochrome cd_1. In the determinations of soluble cytochrome cd_1 the values obtained by purification were higher. However, if the extent of the induction is considered - ie the ratio of nitrate soluble cytochrome cd_1 to aerobic soluble - the ratios are comparable (see Table V) irrespective of the method used. Similarly the level of induction of cytochrome c-551 is comparable.

However, there is a difference in the noted levels of induction of cytochrome c_5.

From gels it has proved impossible to obtain values for the quantity of cytochrome c_4 from nitrate membranes. This is due to lack of resolution of cytochrome c_4 from cytochromes which run just ahead and just behind cytochrome c_4 (see lane (f) of Figures 30 and 31). It can be seen from the scan that cytochrome c_4 is likely to have major contributions from the other 2 cytochromes and any value wil be an overestimate. There was no such problem with aerobic membranes (see lane (b) of Figures 30 and 31) since the band running just ahead of cytochrome c_4 is induced under nitrate conditions. Attempts were made to improve resolution by running gels until the cytochrome c-551 had run off the running gel, but little improvement in resolution was noted.

It was therefore necessary to examine other methods to quantity the cytochrome c_4. To this end Western blotting was attempted.

However, the results of the gel quantitation, for the well resolved cytochromes, have been shown to confirm the conclusions of the purification experiment.

CHAPTER IV : INDUCTION AND CELLULAR LOCATION OF CYTOCHROME c_4

Section III - Quantitation of Cytochrome c_4 from Western Blots

The last section demonstrated that cytochrome c_4 was not well resolved from other c-type cytochromes, making quantitation impossible. By Western blotting, the electophoretic transfer of proteins from polyacrylamide gels to nitrocellulose, and probing with specific anti-c_4 antibody the only stained band visible should be that of cytochrome c_4. Thus quantitation of cytochrome c_4 should be possible from densitometer scans of the nitrocellulose.

Rabbit anti-c_4 antibodies were prepared against cytochrome c_4 purified from membranes of nitrate grown cells and ELISA (see section IV, this chapter) demonstrated that this antibody would react with equal intensity against cytochromes c_4 of soluble/membrane fractions from both aerobic and nitrate grown cells.

A. Demonstration that the colour yield increases linearly with increasing loadings of cytochrome c_4

The properties section (Chapter III section B) demonstrated that cytochrome c_4 reduced and then treated with SDS before application to the gel (porphyrin form) is more antigenic than cytochrome c_4 which was oxidised before the addition of SDS. Therefore for purposes of

quantitation of cytochrome c_4 from extracts it is important to have all the cytochrome in the one state of oxidation/reduction. The fully reduced state was chosen since the colour yield is greater.

Figure 32 shows the colour yield of the HRP reaction over the range of 175-1400ng loading of protein (cytochrome c_4). The second trace (upper) shows the haem staining colour yield over the same range of cytochrome c_4 loading (oxidised form for haem staining).

It can be seen that the haem stain curve is linear up to 875ng after which the colour yield begins to plateau. However, over the whole range of loadings used the colour yield on the Western blot is linear.

From the results of the haem stain quantitation experiment it can be seen that the amount of cytochrome c_4 in the periplasmic fraction sample will be lower than the 175ng loading. The standard cytochrome c_4 loading on these gels was 140ng and the haem stain colour yield for the periplasmic fraction was much lower. Therefore another linearity curve was produced for protein loadings between 35-280ng (i.e. the loadings overlap the first set) and this is shown in Figure 33. Again reduced samples were used for blotting and oxidised for haem staining. This shows that the haem stain colour yield increases linearly with increasing cytochrome c_4 loadings. However, the blotting results show linearity from 140ng right up to 1400ng. Below 140ng protein the curve is no longer linear. This means that the blotting method can only be used for loadings greater than 140ng

Figure 32 : Plot of Area Under Peak Against Loading of Protein (175 - 1400 ng range)

Areas obtained from densitometer scans of nitrocellulose. Plot for haem stained gel with same loadings of protein. Areas are expressed in arbitary units. ●——● haem stain o----o blot.

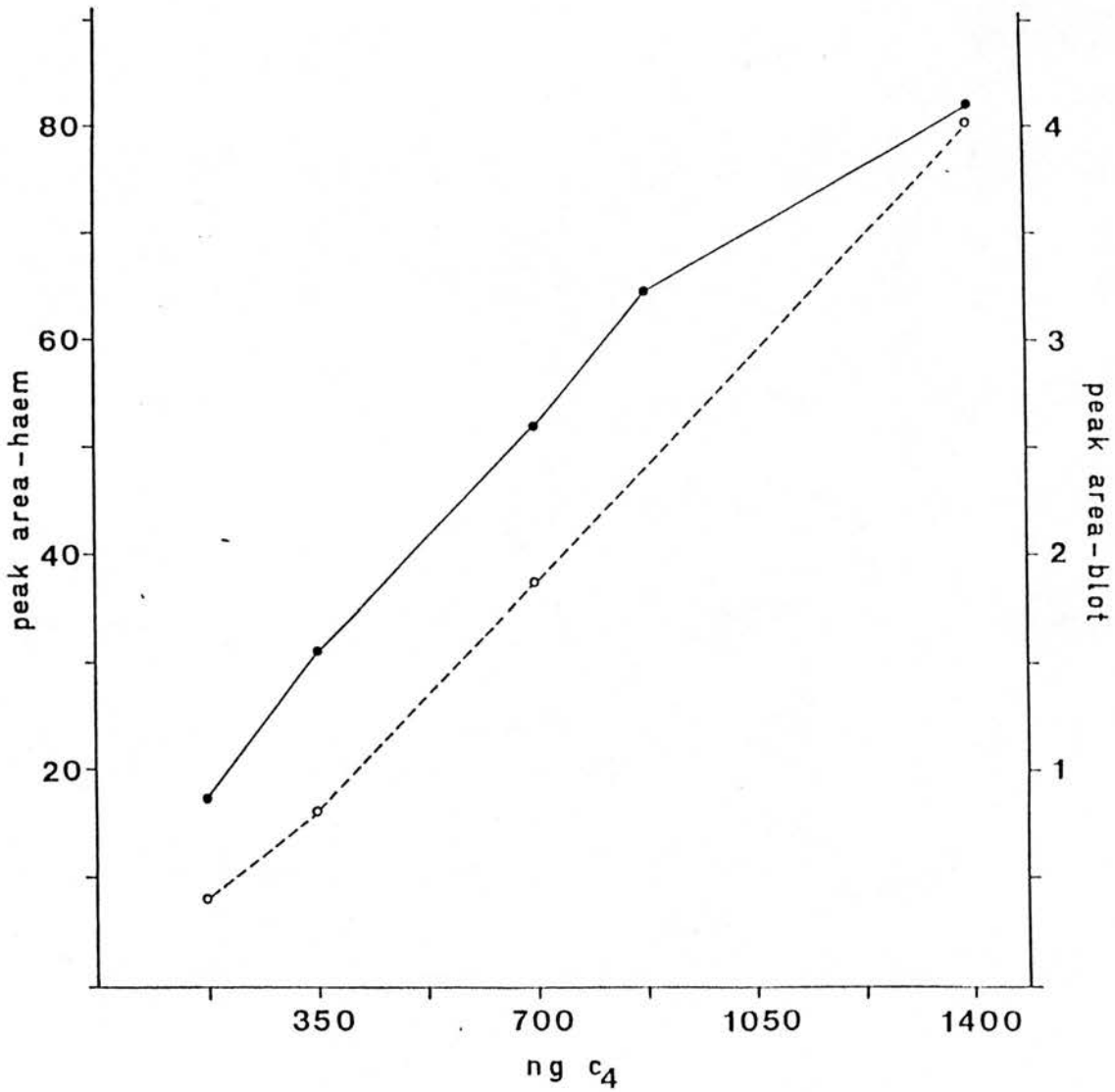
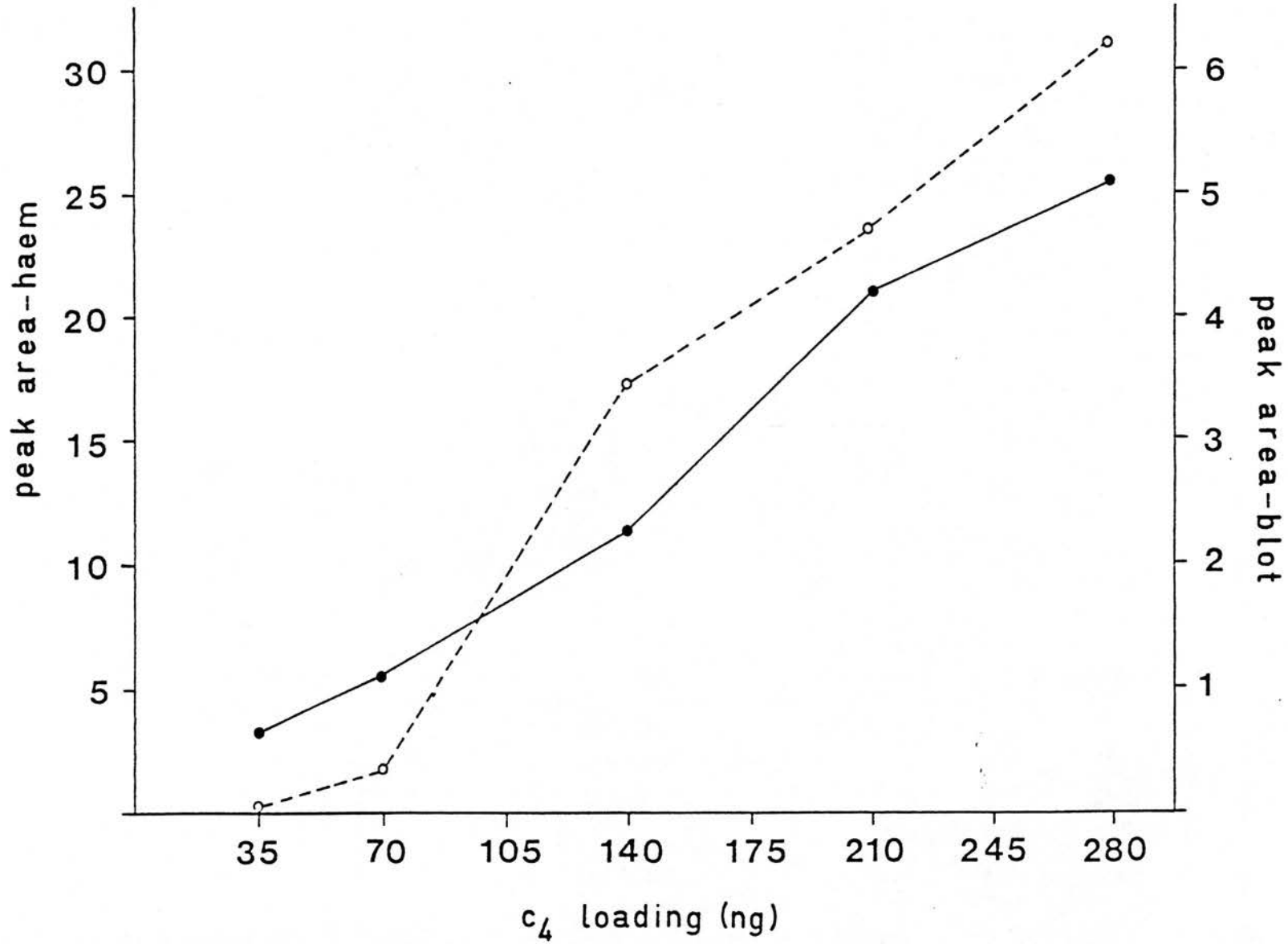

80
60
40
20
peak area-haem
4
3
2
1
peak area-blot
350
700
1050
1400
ng c_4

Figure 33 : Plot of Area Under Peak Against Loading of Protein (35-280ng range)

Areas were obtained from densitometer scans of nitrocellulose. Plot for haem stain used same loading of protein. Areas are expressed in arbitary units. ●—● haem stain, o---o blot.


peak area-haem
30
25
20
15
10
5
peak area-blot
6
5
4
3
2
1
35
70
105
140
175
210
245
280
c_4 loading (ng)

protein. This means that periplasmic cytochrome c_4 cannot be quantitated by this method. Loadings of greater than 140ng protein can be successfully assessed. From the haem stain quantitation gels the colour yield of cytochrome c_4 in the membranes is much greater than the amount of staining for the 140ng standard loading and therefore membrane cytochrome c_4 can be quantitated by Western blotting, which was the main purpose of blotting.

(B) Quantitation of cytochrome c_4 from Western blots

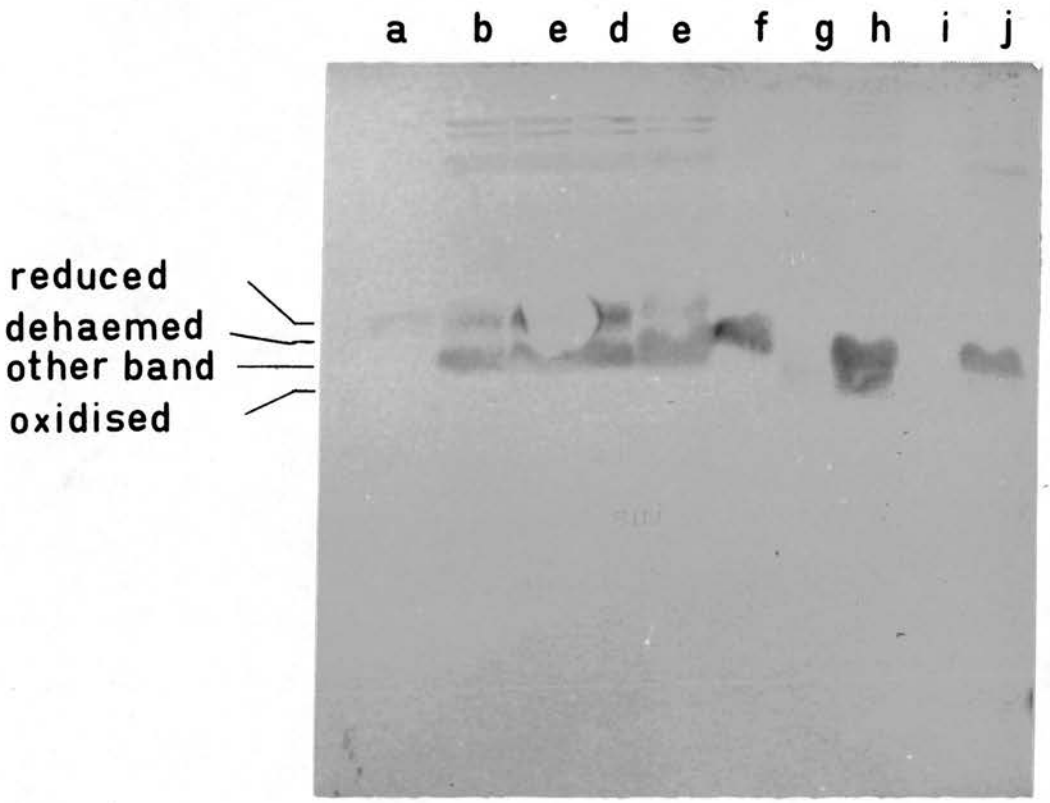
During the course of the blotting experiments it was noted that cytochrome c_4 was not the only protein species that was found to be reactive using the anti-stutzeri c_4 antibody and that the other band highlighted ran with a mobility similar to that of cytochrome c_4 (see lanes b-e of Figure 34). It can be seen that the upper of the two major bands runs with a mobility the same as purified "reduced" cytochrome c_4. It was originally thought that the lower molecular weight band may be cytochrome c_4 in a different oxidation state. The gel in Figure 34 was designed to test whether the lower molecular weight band could be converted into the higher molecular weight form.

Lanes (a) and (g) show the purified cytochrome c_4 treated with mercaptoethanol and ferricyanide respectively (showing the same mobility pattern as discussed in Chapter III). Lane (f) is a sample of dehaemed (apo-) cytochrome c_4. Lanes (b)-(e) have been loaded with aerobic membrane samples treated with mercaptoethanol (see figure legend for details). This shows that none of the mercaptoethanol treatments

Figure 34 : Western Blot of Aerobic Membrane Samples and an Attempt to Identify the Second Stained band

Lane (a) 0.21µg cytochrome c_4[1], (b) aerobic membranes[1], (c) aerobic membranes[2], (d) aerobic membranes plus twice the amount of mercaptoethanol[2], (e) aerobic membranes[1], (f) 350ng dehaemed cytochrome c_4[3], (g) 0.21µg cytochrome c_4 plus 15µg BSA (giving total protein equal to membrane protein loadings thus equalising the protein:SDS ratio)[3], (h) 0.21µg cytochrome c_4 plus aerobic membranes[3], (i) 0.21µg cytochrome c_4[3] and (j) aerobic membranes[3].

Gel sample treatments: (1) mercaptoethanol added (to 1.25 or 2.5mM), left for two mins then Laemmli buffer added followed by a two min boil, (2) mercaptoethanol added, left for two mins then Laemmli buffer added followed by a one hour incubation at 37_oC and (3) potassium ferricyanide added (to 1.25mM), left for two mins then Laemmli buffer added followed by a two min boil.


a b e d e f g h i j
reduced
dehaemed
other band
oxidised

successfully converts the lower molecular weight band to the "reduced" cytochrome c_4 form. As a double check lane (i) was treated with ferricyanide to attempt conversion to the "oxidised" cytochrome c_4 form. This lane shows that the position of the cytochrome c_4 has been shifted from above the other band to below it. It should be noted that although the membrane loadings were equivalent the intensity of the cytochrome c_4 in the ferricyanide treated samples is much less than the mercaptoethanol treated samples suggesting that the "reduced" (fully unfolded (see Chapter III Section B) form) is more antigenic. To demonstrate the position of the ferricyanide treated cytochrome c_4 more clearly lane (h) was loaded with membranes plus purified cytochrome c_4 and this is seen to run in front of the other band. This gel also demonstrates that the other band does not have the same mobility as apo-cytochrome c_4.

It can therefore be concluded that from the pairs of bands in the membrane samples, one of them is cytochrome c_4 and the other is not. It was also noted in previous experiments (not shown) that this other band is present in all 3 cellular compartments and it has since been shown (G.W.Pettigrew) that this other band corresponds to a major membrane protein which is not a c-type cytochrome.

(C) Conclusions from the blotting experiments

The antiserum prepared in the rabbits is not suitable for quantitation experiments from cell extracts since it also contains an antibody against a major

membrane protein which runs with almost the same mobility as cytochrome c_4. Again the cytochrome c_4 band is not well resolved from the major band making quantitation impossible. This major band has been shown not to be cytochrome c_4 and is also not the c-type cytochrome which runs just ahead of cytochrome c_4 in nitrate membranes (see Figure 30) since the major band is present in all fractions and the other c-type cytochrome is induced under nitrate growth conditions. These results suggest that blotting will prove useful in quantitation experiments providing a "clean" antiserum can be produced. It may have been that the rabbits had been exposed to Pseudomonas before injection of the cytochrome c_4 antigen. The antibody would have to be produced in germ-free rabbits or alternatively monoclonal antibodies prepared.

The antiserum is still useful providing purified cytochrome c_4 is used. i.e. ELISA results were obtained from this antiserum.

CHAPTER IV : INDUCTION AND CELLULAR LOCATION OF CYTOCHROME c_4

Section IV - Comparison of Cytochromes c_4 Purified from Membrane/Soluble Fractions of Aerobic and Nitrate Grown Cells

The quantitation experiments demonstrated that cytochrome c_4 is found in both aerobic and nitrate grown cells and also that it is both membrane bound and found in the soluble fraction. It is therefore essential to show whether (i) the soluble (S) and membrane (M) forms are identical and (ii) whether the aerobic (A) and nitrate (N) forms are also identical. Four cytochrome c_4 preparations -AS,AM,NS,NM- are compared.

A. Spectra

Spectra of the dithionite reduced cytochromes c_4 were recorded between 580 and 510nm and are shown in Figure 35. These show that the 4 cytochromes c_4 samples have α-peak maxima at 550nm and β-peak maxima at 522nm. The α/β ratios are also virtually identical.

B. ELISA

Antibodies prepared against cytochrome c_4 purified from membranes of nitrate grown cells were tested against the 4 batches of cytochrome c_4. Figure 36 shows pairs of ELISA results plotted. It is important to note that the 4 cytochromes c_4 were tested on the same ELISA microtitre plate thus enabling a direct comparison to be made. The plots show that for each cytochrome c_4 batch

Figure 35 : Dithionite reduced Spectra in the Region of the α- and β-Bands for the Four Cytochrome c_4 Preparations

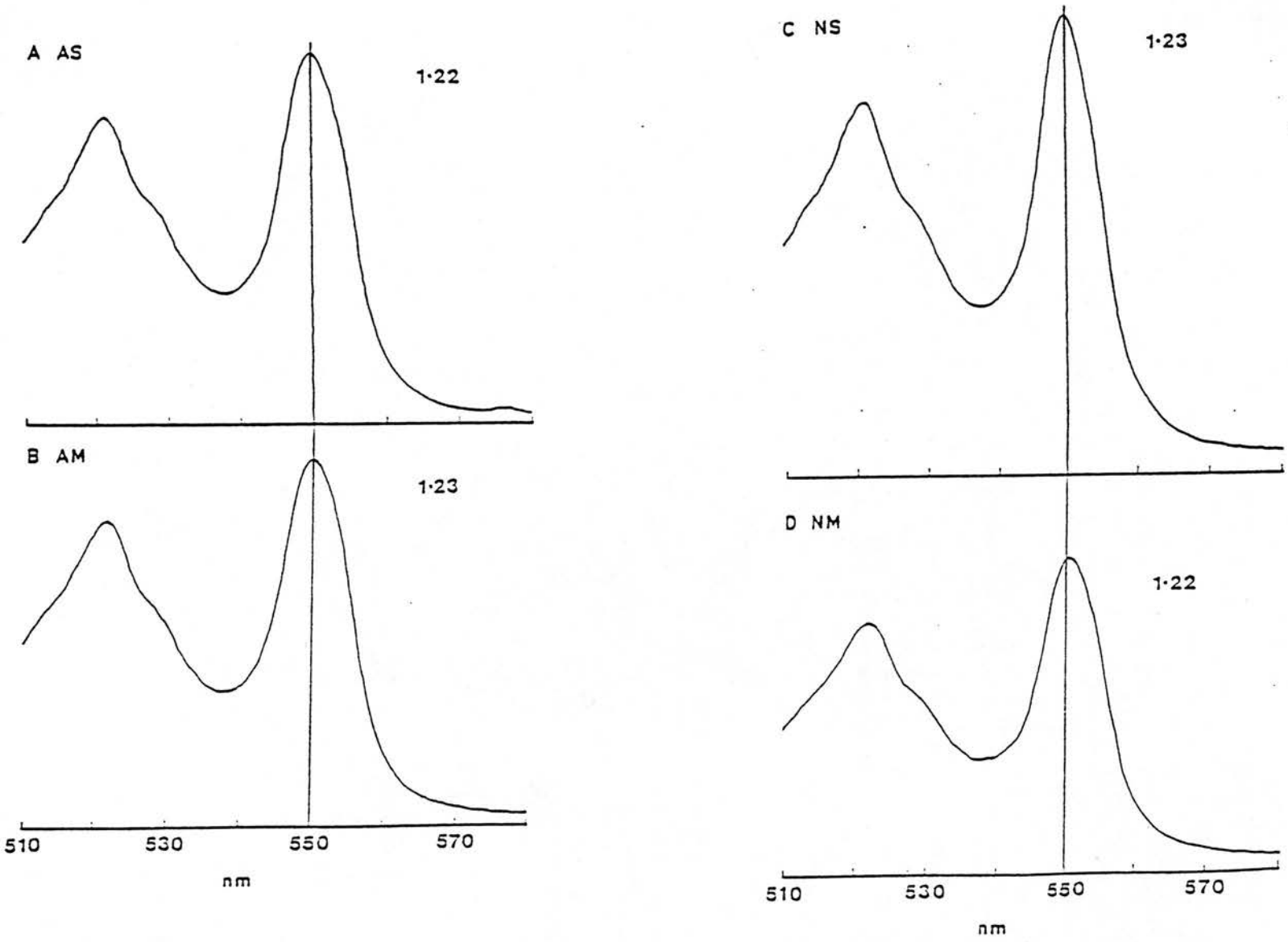

A AS
1·22
B AM
1·23
510
530
550
570
nm
C NS
1·23
D NM
1·22
510
530
550
570
nm

Figure 36 : Comparison of the 4 Cytochrome c_4 Preparations by ELISA

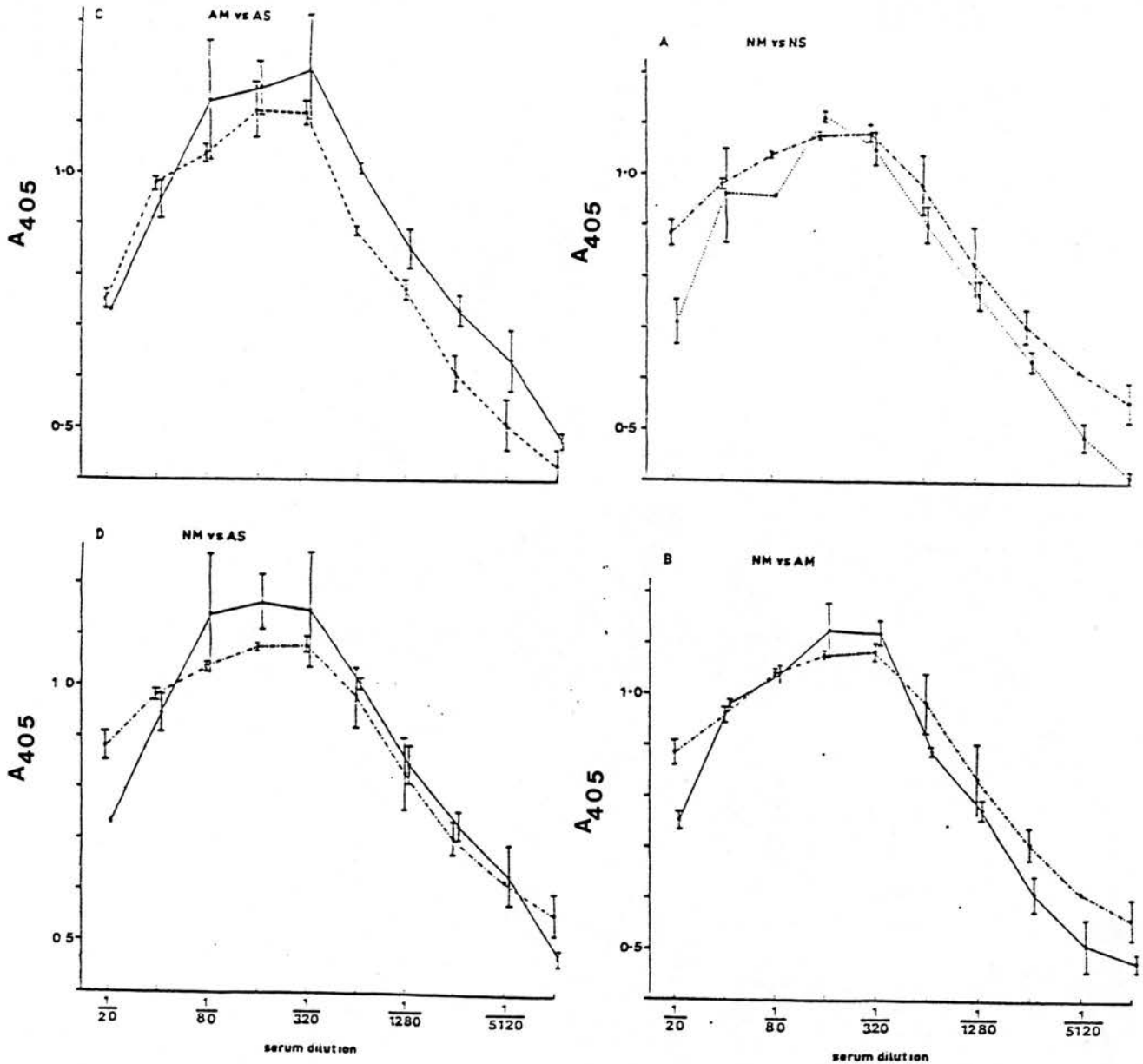
AS - - - - -

AM ————

NS .....

NM —·—·—·—·

Bars join two experimental points and the line is drawn through the average of these.


C
AM vs AS
A
NM vs NS
D
NM vs AS
B
NM vs AM
A405
1·0
0·5
1 0
0 5
1/20
1/80
1/320
1/1280
1/5120
serum dilution

the maximum response is noted over the same dilution range in each case.

Secondly, the magnitude of the maximal response is the same in each case; and thirdly, the response begins to diminish at the same dilution factor of antibody in each case. These results strongly suggest that the 4 batches of cytochrome c_4 are identical.

C. Redox titration

Table VI shows the results of titration of each batch. These results show that there is no substantial difference between the cytochromes c_4.

D. Amino acid analysis

The results of the analyses are presented in Table VII and in general the analyses are very similar. In addition the results were compared by the Cornish-Bowden method (see Table VIII) which indicated that the percentage differences between them are very low.

E. Summary

Taken together these results suggest that the cytochromes c_4 from membrane/soluble fractions from both aerobic and nitrate grown cells are identical.

Table VI : Redox Titration of the Four Preparations of Cytocrome c_4

Aerobic Soluble	+300 & +210mV
Aerobic Membrane	+300 & +190mV
Nitrate Soluble	+300 & +200mV
Nitrate Membrane	+300 & +210mV

Redox titration analysis carried out at 550nm.

Table VII : Amino Acid Compositions of the Four Cytochrome c_4 Preparations

Figures are expressed as moles percent

	AS	AM	NS	NM
Asp	11.82	11.20	11.61	12.25
Thr	3.62	3.63	4.32	3.85
Ser	4.84	4.80	4.81	4.91
Glu	11.00	11.35	12.92	10.79
Pro	6.87	4.88	4.99	5.03
Gly	13.16	13.22	11.74	14.01
Ala	13.06	12.88	12.53	13.19
Val	5.26	3.55	4.64	3.70
Met	2.34	2.37	2.40	2.51
Ile	2.86	3.41	3.66	3.02
Leu	8.32	10.04	8.08	8.98
Tyr	3.21	3.57	3.07	3.49
Phe	2.15	2.58	2.57	2.32
His	2.53	2.56	2.39	2.32
Lys	6.29	6.92	6.74	6.59
Arg	2.65	3.03	3.52	3.02

Table VIII : Cornish-Bowden Analysis of the Amino Acid Compositions of the Four Cytochrome c_4 Preparations

	AS	AM	NS	NM
AS	X	7	7	5
AM	7	X	8	4
NS	7	8	X	7
NM	5	4	7	X

CHAPTER V : MEMBRANE "SIDEDNESS" OF CYTOCHROME c_4

The quantitation experiments have demonstrated that cytochrome c_4 is predominantly associated with the membrane but give no indication to which side (cytoplasmic or periplasmic) of the membrane it is attached to. To enable such a determination cytochrome c_4 must be examined while it is still attached to the membrane. One such method for determining the "sidedness" of a membrane protein involves exposing either the external or the cytoplasmic surface of the membrane to proteolytic enzymes and assess, by SDS-PAGE, which protein bands have been removed by this treatment. For these experiments it is essential to produce membrane vesicles which are either right-side-out or inside-out. For the purpose of cytochrome c_4 location right-side-out vesicles (intact spheroplasts) were used.

A. Preparation of membrane vesicles

These experiments were carried out using aerobic membranes because (1) more cytochrome c_4 is associated with aerobic membranes and (2) cytochrome c_4 is poorly resolved on SDS-PAGE in nitrate membranes.

Membrane vesicles (spheroplasts) were prepared by the lysozyme/EDTA treatment of cells (see Chapter IV Section II). However, for the purpose of these experiments the centrifugation to produce a spheroplast pellet was omitted. It was found that resuspension of the spheroplasts to homogeneity in sucrose medium was

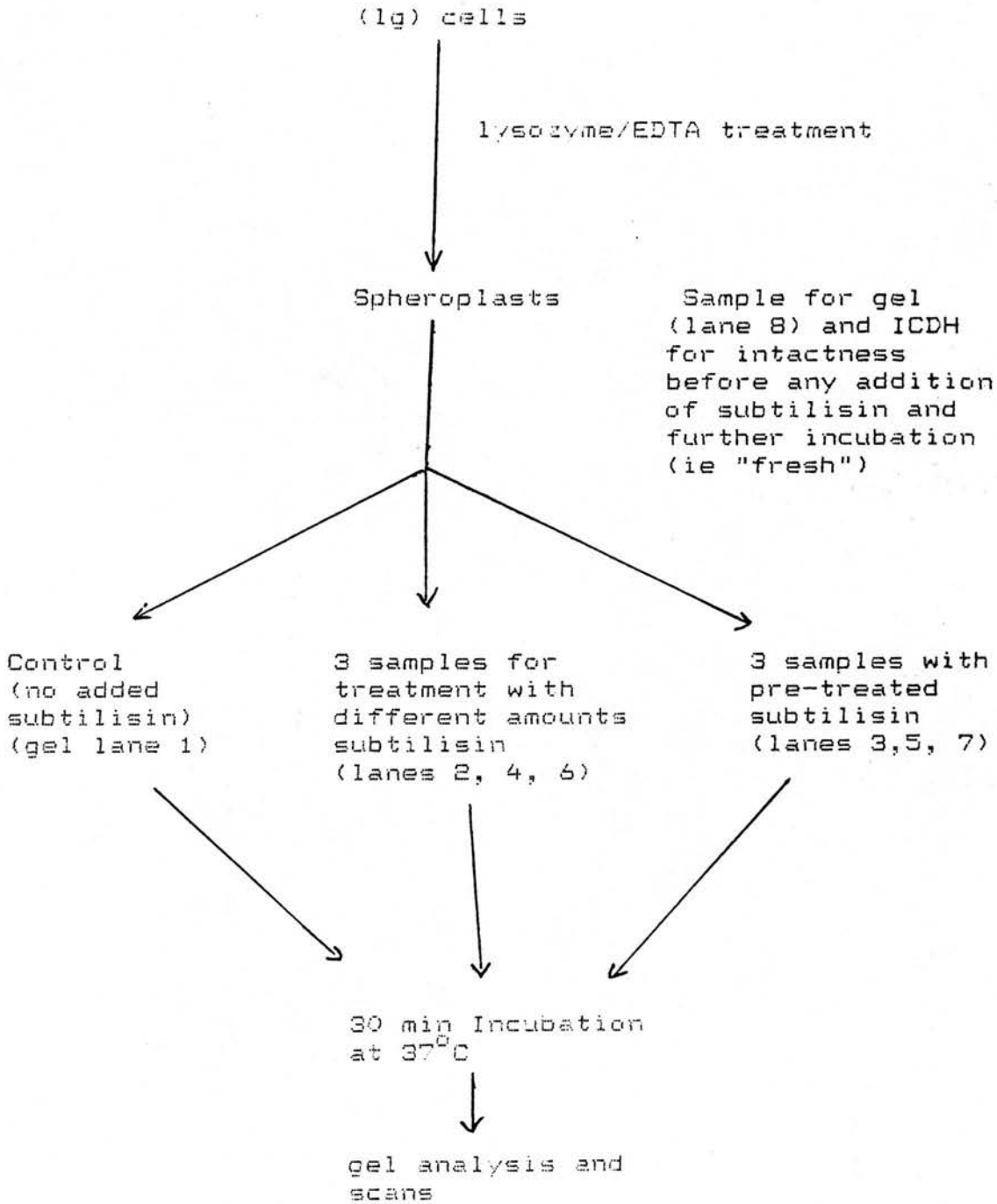
difficult. Thus digestion was carried out on the unfractionated spheroplast suspension.

Assessment of spheroplast intactness was determined immediately after the 30 min incubation time with lysozyme/EDTA (see Figure 37). A small sample of the spheroplast mixture was taken, the periplasm separated from the spheroplasts by centrifugation and the spheroplasts lysed. ICDH (a cytoplasmic enzyme) activity was measured in the periplasm and the spheroplast lysate. The percentage of the ICDH activity found in the periplasmic fraction indicates the amount of spheroplast lysis. It was found that the 1.4% of the total ICDH was associated with the periplasm implying that 98.6% of the spheroplasts were intact. This value of 98.6% corresponds to the intactness of the vesicles before treatment with protease (ie immediately after lysozyme/EDTA treatment).

B. The treatment of spheroplasts with subtilisin

This method is described schematically in Figure 37. To 0.1g (wet weight) spheroplasts was added subtilisin : "accessible" protein ratios of 0.05, 0.01 and 0.004 by weight. The quantity of "accessible" protein used for this calculation included periplasmic protein + added lysozyme + membrane protein. However, not all of the membrane protein will be accessible to protease so these ratios are underestimates. Subtilisin treatment was allowed to continue for 30 mins at 37^{o}C after which the protease was inhibited by addition of a 30 fold molar excess of PMSF. For each ratio of subtilisin to protein

Figure 37 : Scheme for Membrane "Sidedness" Experiments



a control tube was set up and incubated as above but with subtilisin pretreated with PMSF.

The samples were then spun to pellet the spheroplasts. The spheroplasts were resuspended (with lysis) in 10mM phosphate and analysed on haem-stained gels. Samples from the periplasmic fraction and lysed spheroplast fraction were analysed for ICDH.

C. Analysis of membranes on SDS-PAGE stained for haem

(i) Investigation of endogenous protease activity

Densitometer scans of lanes a and h of the gel shown in Figure 38 were recorded. Lane h is a sample of "fresh" spheroplasts (refer to Figure 37) and lane 1 is a spheroplast sample incubated at 37°C for 30 mins in the absence of subtilisin. Therefore comparison of peak areas will show the presence or absence of any endogenous protease. The results in Table IX show that there is little difference (ie < 10% variation) in the areas under the peaks for band I, band II and cytochrome c_4 which implies that no endogenous protease is active to affect future results.

(ii) Effect of subtilisin on membrane bound cytochrome c_4

Qualitatively, Figure 38 lanes b, d and f show that increasing the ratio of subtilisin to protein progressively removes more cytochrome c_4 from the membrane - ie complete removal of the cytochrome c_4 at the 0.05 ratio. It should also be noted that 2 lower molecular weight bands are produced by subtilisin activity which are missing in the pretreated subtilisin samples indicating that PMSF is a very effective

Figure 38 : SDS-PAGE Analysis of Removal of Cytochrome c_4 from Aerobic Membranes by Subtilisin

Lanes (a) to (g) show aerobic spheroplast samples incubated at 30°C for 30mins at the subtilisin/protein ratio and PMSF treatments as shown by the gel labelling (and also described in the text). Lane (h) was loaded with "fresh" spheroplasts.

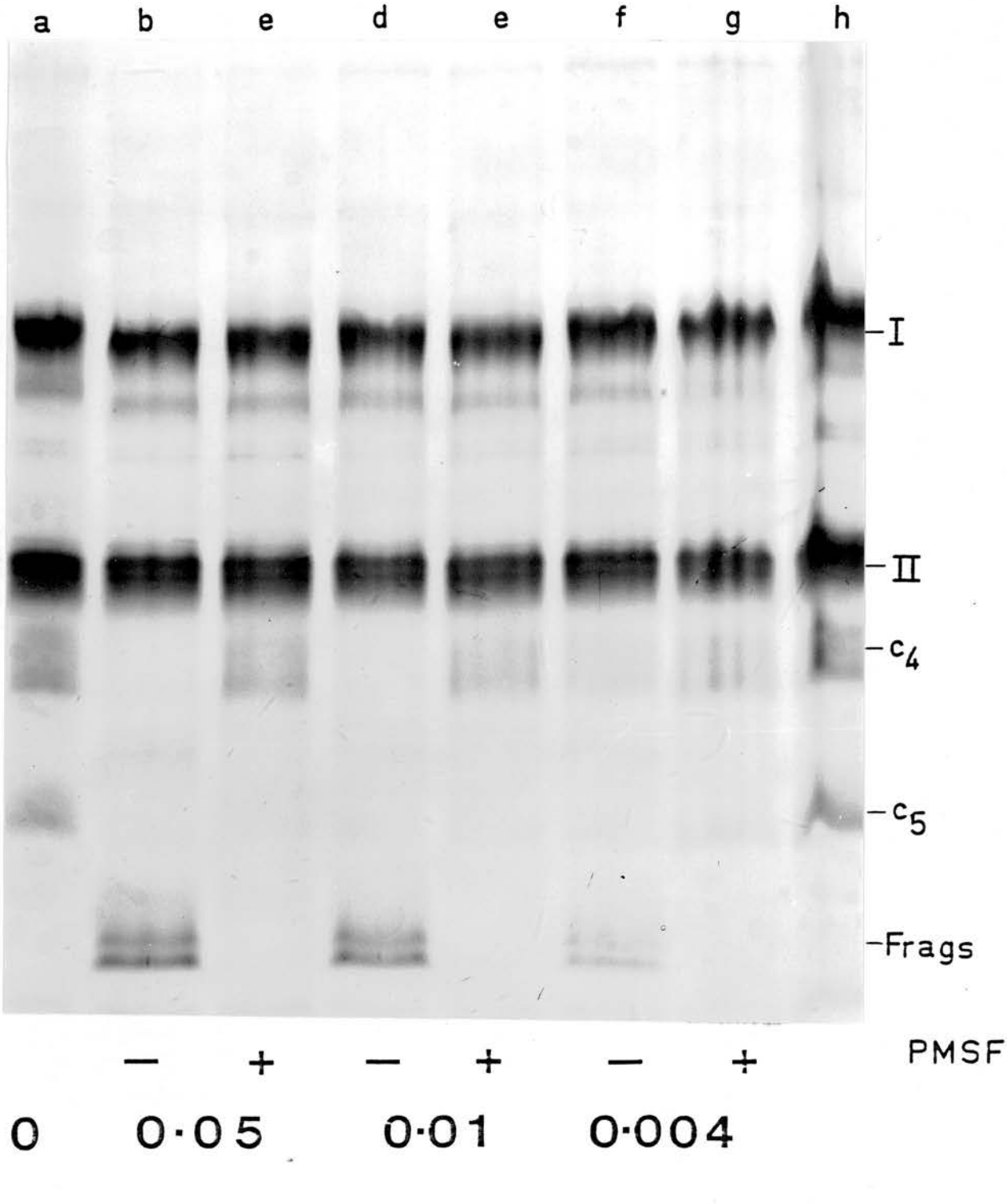

a
b
e
d
e
f
g
h
I
II
c_4
c_5
Frags
—
+
—
+
—
+
PMSF
0
0·05
0·01
0·004
subtilisin/protein

Table IX : Effect of Incubation of Intact Spheroplasts at 37°C for 30 mins with no Subtilisin. Control for this Experiment was Membranes Taken Immediately after the Lysozyme/EDTA Treatment (see Figure 38 for details). Values are Areas Under Peaks from Densitometer Scans (Arbitary Units)

Peak	Fresh	30 min incubation
Band I	19.6	20.0
Band II	16.4	13.3
Cytochrome c_4	6.1	5.7

inhibitor of subtilisin. Experiments (not shown) with purified cytochrome c_4 show that subtilisin is very effective (100%) in digesting cytochrome c_4 but if subtilisin was pre-treated with PMSF no digestion of cytochrome c_4 was noted.

Table X shows the results of a quantitative study of loss of cytochrome c_4 from the membrane at the 0.05 ratio. This ratio was selected, since it appears from the gel that cytochrome c_4 was completely removed by the subtilisin. The results indicate that 47% of the cytochrome c_4 was removed from the membranes in the pre-treated subtilisin tube. As has been stated above, PMSF is 100% effective in inhibiting subtilisin. In this case it may be the effect of propanol on the membrane - ie acting in the same way as butanol does in the purification method. This implies that the addition of propanol at the end of the incubation period could be responsible for removal of 47%. It is important to note that in the tube with active subtilisin propanol is absent during the 30 min incubation, and therefore this extraction is not happening during this time. One additional control should have been done: the effect of the time exposure to propanol on the loss of cytochrome c_4 from the membrane. After addition of PMSF at the end of the 30 min incubation time the tubes were spun immediately thus the exposure time is much shorter than for pre-treated subtilisin samples.

However, it is essential to stress that a minimum of

Table X : Analysis of Removal of Cytochrome c_4 from Membranes

Areas were determined from densitometer scans of lanes a-c from the gel shown in Figure 38. Values expressed in arbitary units. Percentage cytochrome c_4 is expressed against the quantity of c_4 found in lane a (zero subtilisin)

Subtilisin Protein	pre-PMSF	peak area	% c_4
0	-	5.7	100
0.05	-	0.4	7
0.05	+	3.0	53

46% cytochrome c_4 removal can be accounted for by subtilisin. It should also be noted that the method of removal of the cytochrome c_4 from the membrane is not important (ie whether subtilisin and/or propanol removes the cytochrome c_4) as long as the spheroplasts remain intact. Therefore if the spheroplasts are intact and the combination of subtilisin and propanol (ie the propanol is added with the PMSF to inhibit the subtilisin at the end of the incubation period) removes 93% of the cytochrome c_4 (Table X) then it can be stated that the cytochrome c_4 is located on the periplasmic face of the membrane.

D. Assessment of intactness of the spheroplast vesicles

Before a statement can be made about the sidedness of cytochrome c_4 it must be shown that the spheroplasts have remained intact - ie allowing protease exposure to the periplasmic face of the membrane only. This is achieved by assessing the release of isocitrate dehydrogenase (a cytoplasmic enzyme) into the periplasmic fraction and/or by assessing the amount of ICDH within the spheroplasts. It is obvious that any released ICDH will come into contact with the subtilisin, which may proteolytically inactivate it, and also the propanol/PMSF which may inhibit its activity. It is therefore essential to assess the effect of subtilisin and PMSF/propanol on the ICDH enzyme.

(i) Effect of subtilisin and PMSF/propanol on ICDH activity

Cytoplasmic fractions of Pseudomonas stutzeri were prepared as described in Chapter IV Section II. Experiments were set up to test the effect of subtilisin, propanol and propanol/PMSF on the ICDH activity. A subtilisin:protein ratio of 0.05 was used since this was the highest ratio used to remove cytochrome c_4 from the membranes. At the end of the incubation period no inhibitor was added; instead the ICDH assay for this sample was carried out immediately. Propanol alone was added to 4% (V/V) to demonstrate any effect of propanol and lastly an amount of PMSF (in propanol) was added to a cytoplasmic sample equivalent to the amount required to inhibit subtilisin at 0.05 ratio (here propanol was again 4%). The control had distilled water added and all tubes were a constant volume. Incubation was carried out for 30 mins at $37^{o}C$.

Table XI shows the results of the ICDH assays. The results indicate that propanol and PMSF have no effect on the ICDH activity. However, subtilisin inactivated 66% of the ICDH. This means that assessment of periplasmic ICDH cannot be used as a means of estimating spheroplast lysis. Therefore the ICDH activity remaining inside the spheroplasts must be assayed and used to calculate the extent of lysis.

(ii) Assessment of intactness of spheroplasts after incubation period

Again the results shown will be for the 0.05 ratio of subtilisin:protein. Table XII shows the results of the ICDH assays. The percentage intactness is expressed

Table XI : Effect of Subtilisin, Propanol and PMSF on ICDH Activity

Tube Contents	% ICDH activity remaining
Cytoplasm alone	100
Cytoplasm + subtilisin	34
Cytoplasm + propanol	100
Cytoplasm + PMSF/ propanol	95

Table XII : Intactness of Spheroplasts

The % intactness of the samples with subtilisin was calculated as follows: $\frac{\text{cytoplasmic ICDH}}{\text{zero subtilisin total}}$ %

Subtilisin protein	pre-PMSF	cyto-plasmic	peri-plasmic	total	% intactness
0	-	440	5	445	98.9
0.05	-	370	13	383	83
0.05	+	370	14	384	83

relative to the tube with zero subtilisin. It can be seen that the percentage intactness is still high (83%) after incubation. It can be concluded that since almost all the cytochrome c_4 is removed from the membrane by the action of subtilisin treatment (which includes treatment with propanol) and 83% of the spheroplasts remained intact, thus allowing subtilisin access to only the periplasmic face of the membrane, the cytochrome c_4 must be located on the periplasmic side of the membrane.

CHAPTER VI : REDOX TITRATION OF CYTOCHROME C_4

Section I - The Nernst Equation

An important physical property of an electron transfer protein is its redox midpoint potential. The midpoint potential may reveal some information on the possible position of the electron transfer protein within the electron transfer chain (ie the more positive the midpoint potential the nearer the terminal acceptor it is likely to be). Also individual c-type cytochromes may have more than one haem. For example cytochrome c_4 with 2 (Swank and Burris, 1969), cytochrome c-554 from Nitrosomonas europaea with 4 (Andersson et al, 1986). Redox potentiometry may reveal if these haems have identical midpoint potentials or if each haem has a different potential. It is therefore important to discuss the method of analysing redox data and deciding on the number of components a system has. The most important equation to apply to redox potentiometry is the Nernst equation and therefore the derivation of the equation will be presented.

Consider the following half cell

$$A_{red} \rightleftharpoons A_{ox} + ne^- \qquad (1)$$

where n is the number of electrons given up by A_{red}. As electrons are unable to exist in a free state there must be another half cell reaction to accept electrons.

$$B_{ox} + ne^- \rightleftharpoons B_{red}$$

(2)

The overall reaction can be described as

$$A_{red} + B_{ox} \rightleftharpoons A_{ox} + B_{red} \qquad (3)$$

As with all equilibrium reactions an equilibrium constant can be calculated. The equilibrium constant for equation (3) can be expressed as follows

$$K_{eq} = \frac{[A_{ox}]\ [B_{red}]}{[A_{red}]\ [B_{ox}]} \qquad (4)$$

Consequently the standard free energy of the reaction can be calculated.

$$\Delta G_o = -RT\ln K_{eq} \qquad (5)$$

for non standard conditions expression (5) becomes

$$\Delta G = \Delta G_o + RT\ln \frac{[A_{ox}]\ [B_{red}]}{[A_{red}]\ [B_{ox}]} \qquad (6)$$

Common to all reactions between 2 half cells is the transfer of electrons. For these reactions equation (6) can be expressed in terms of electrical potential units rather than free energy given that

$$\Delta G = -nF\Delta E \qquad (7)$$

where ΔE is the redox difference between the 2 half cells, F is the Faraday (the constant required to convert chemical potential to electrical potential) and n, again, is the number of electrons transferred during the reaction.

Therefore, equation (6) becomes

$$\Delta E = \Delta E_o - \frac{RT}{nF} 1n \frac{[A_{ox}]\ [B_{red}]}{[A_{red}]\ [B_{ox}]} \qquad (8)$$

or expressing (8) in terms of A and B

$$E_{(B)} - E_{(A)} = E_{o(B)} - E_{o(A)} + \frac{RT}{nF} 1n \frac{[B_{ox}]}{[B_{red}]} - \frac{RT}{nF} 1n \frac{[A_{ox}]}{[A_{red}]} \qquad (9)$$

This equation now shows that the ΔE of the redox couples A and B can be calculated. This equation therefore allows the E_o of any half cell to be calculated with respect to all the other half cells. It is therefore desirable to refer the electron affinities of the individual half cells to that of a chosen standard. The chosen standard half cell is the Standard Hydrogen half cell (ie hydrogen gas at 1 atmosphere in equilibrium with a solution of hydrogen ions at unit acitivity, pH O) which has been assigned a potential of zero volts, at any temperature.

If A in equation (9) is the hydrogen half cell then it can be seen that any differences disappear since the

potential of the hydrogen half cell is zero (ie $E_{(A)}$ and $E_{o(A)}$ equal zero. Also

$$[A_{red}] = [A_{ox}] \text{ and therefore } \frac{RT}{nF} 1n \frac{[A_{ox}]}{[A_{red}]} = 0$$

the redox potential of the B half cell is now referred to the hydrogen electrode and equation (9) now becomes

$$E_h = E_o + \frac{RT}{nF} 1n \frac{[B_{ox}]}{[B_{red}]} \qquad (10)$$

where h denotes that the selected reference half cell is the hydrogen half cell, E_o is therefore the standard redox potential of the B half cell at pH O and unit activities.

However, biological conditions are non-standard. The term E_o is replaced by $E_{m,x}$, where m stands for midpoint potential (ie when [ox] = [red] and x the ambient pH.

Titrations of biological systems are usualy carried out at 30^oC. Equation (10) can therefore be simplified to

$$E_h = E_{m,x} + \frac{0.06}{n} \log \frac{[ox]}{[red]} \qquad (11)$$

the Nernst equation. The application of the Nernst equation to a redox titration and determination of the number of redox components will be discussed in the next section.

CHAPTER VI : REDOX TITRATION OF CYTOCHROME C_4

Section II - Redox Titrations of Cytochromes and Application of the Nernst Equation for Data Analysis

During the course of a redox titration the following parameters are measured - (1) the ambient potential (E_h) as measured using an appropriate electrode and (2) the value of log [ox]/[red] which is determined spectrophotometrically.

A. Redox titration of horse heart cytochrome c

Figure 39 shows spectra of cytochrome c recorded during an oxidative titration. Values of log[ox]/[red] were calculated from absorbance changes at the α-peak maximum. Figure 40 shows a plot of log[ox]/[red] vs. E_h giving a midpoint potential of +256mV and a slope of 60mV. From the Nernst Equation this gives a value of n=1 - ie one electron is transferred. This is as expected for a monohaem cytochrome whose redox centre contains one iron undergoing the following one electron oxidation/ reduction.

$$Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$$

The value of +256mV agrees well with the published value (+255mV obtained by Henderson and Rawlinson, (1956)).

Figure 39 : Redox Titration of Horse Heart Cytochrome c

Spectra shown were recorded during an oxidative tiration. Each spectrum corresponds to a particular ambient redox potential. Values of log [ox]/[red] were calculated from these spectra (refer to methods).

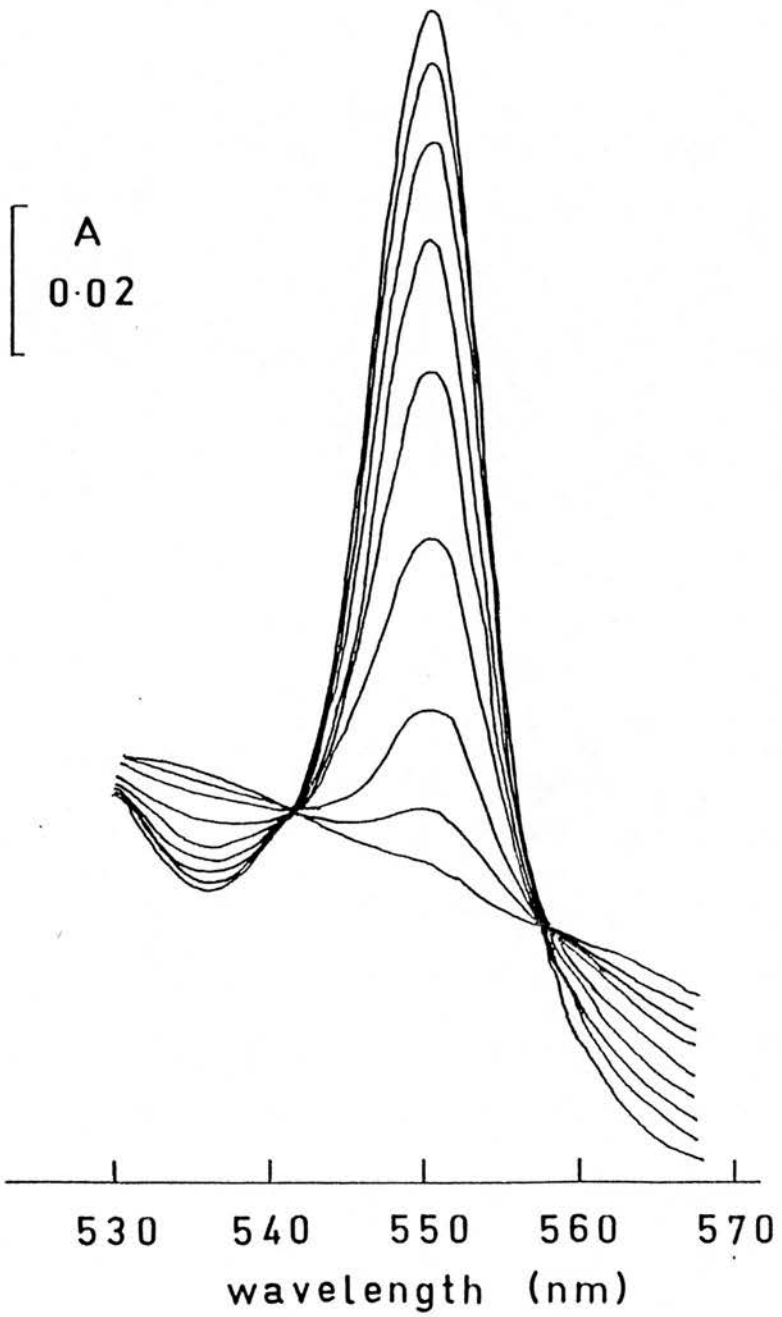
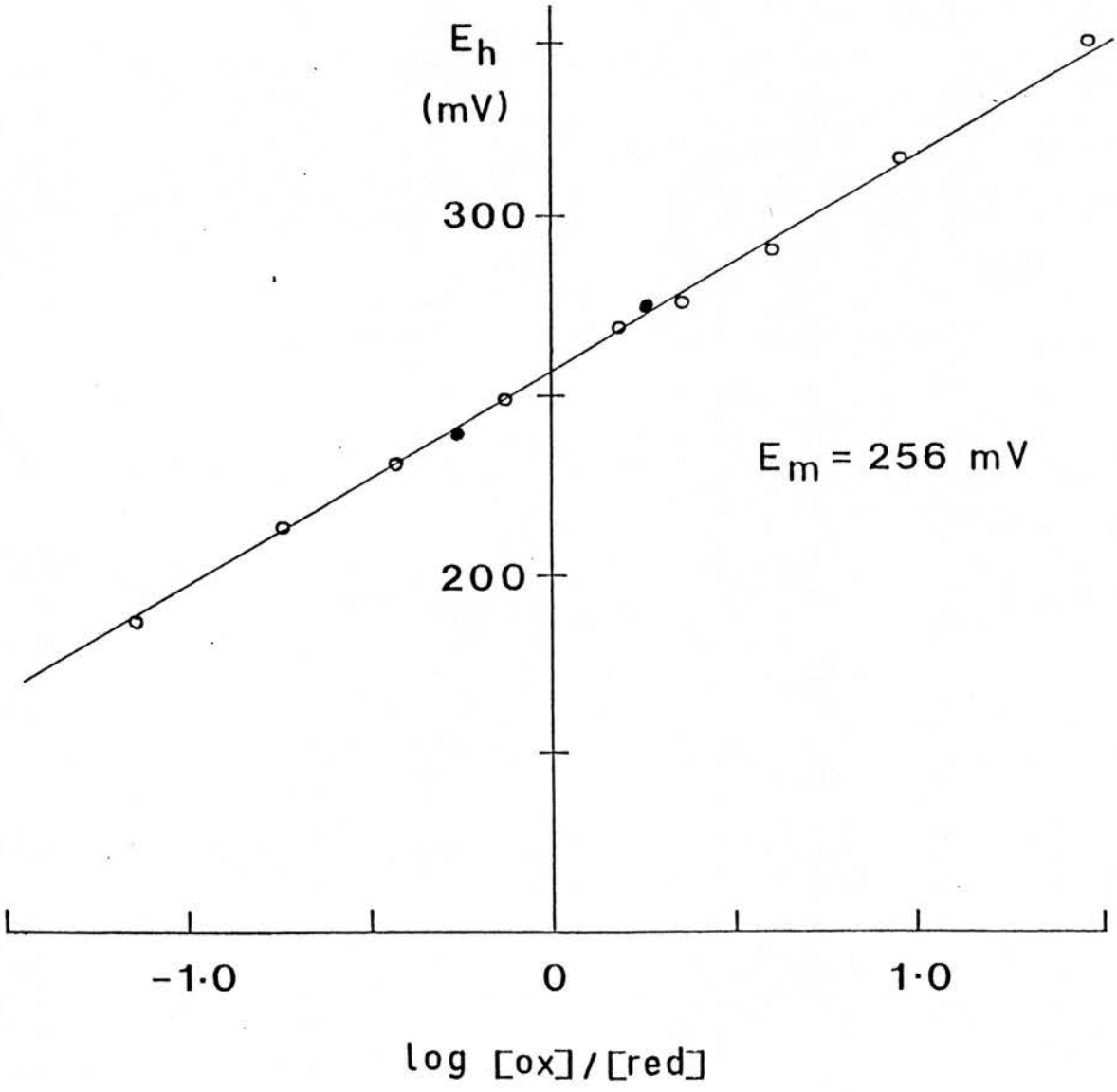

A
0·02
530
540
550
560
570
wavelength (nm)

Figure 40 : Nernst Plot for Horse Heart Cytochrome c

Experimental points, o ● are from oxidative and reductive titrations respectively. Values of log[ox]/[red] were calculated as described in the text. The line shows a slope of 60mV.


E_h
(mV)
300
200
E_m = 256 mV
-1·0
0
1·0
log [ox]/[red]

B. Redox titration of cytochrome c_4 from Pseudomonas stutzeri 224

The spectra recorded during the oxidative titration are shown in Figure 41. These spectra clearly demonstrate the presence of 2 spectrally distinct components. At full reduction the α-peak maximum is 550nm and at partial reduction the α-peak maximum is shifted to 552nm. It can also be seen from these spectra that each spectral component has its own unique isosbestic points (labelled on Figure 41).

Because of the complexity of these spectra it is possible to calculate log[ox]/[red] in 2 ways at each ambient potential. Namely values at the α-peak maximum (variable) or values at 550nm alone. Figure 42(a) shows the Nernst plot of the analysis at 550nm. Clearly these points do not lie on a 60mV slope but instead show a sigmoidal shape indicative of a 2 component system (Wilson and Dutton, 1970). These workers demonstrated that mixing 2 monohaem c-type cytochromes of different redox potentials and titrating the resulting mixture sigmoidal Nernst plots were obtained. These sigmoidal plots can be resolved arithmetically into two components in the following way. At the inflexion point it was assumed that the "lower" (ie the more negative redox potential) component was 100% oxidised and the "upper" component 100% reduced (ie the more positive redox potential). Using this information, and also the relative contribution of each component (estimated from the position of the inflexion point with respect to the

Figure 41 : Spectra Recorded During an Oxidative Titration of Cytochrome c_4

The arrows show the positions of the two isosbestic points (ie one for each contributing component)

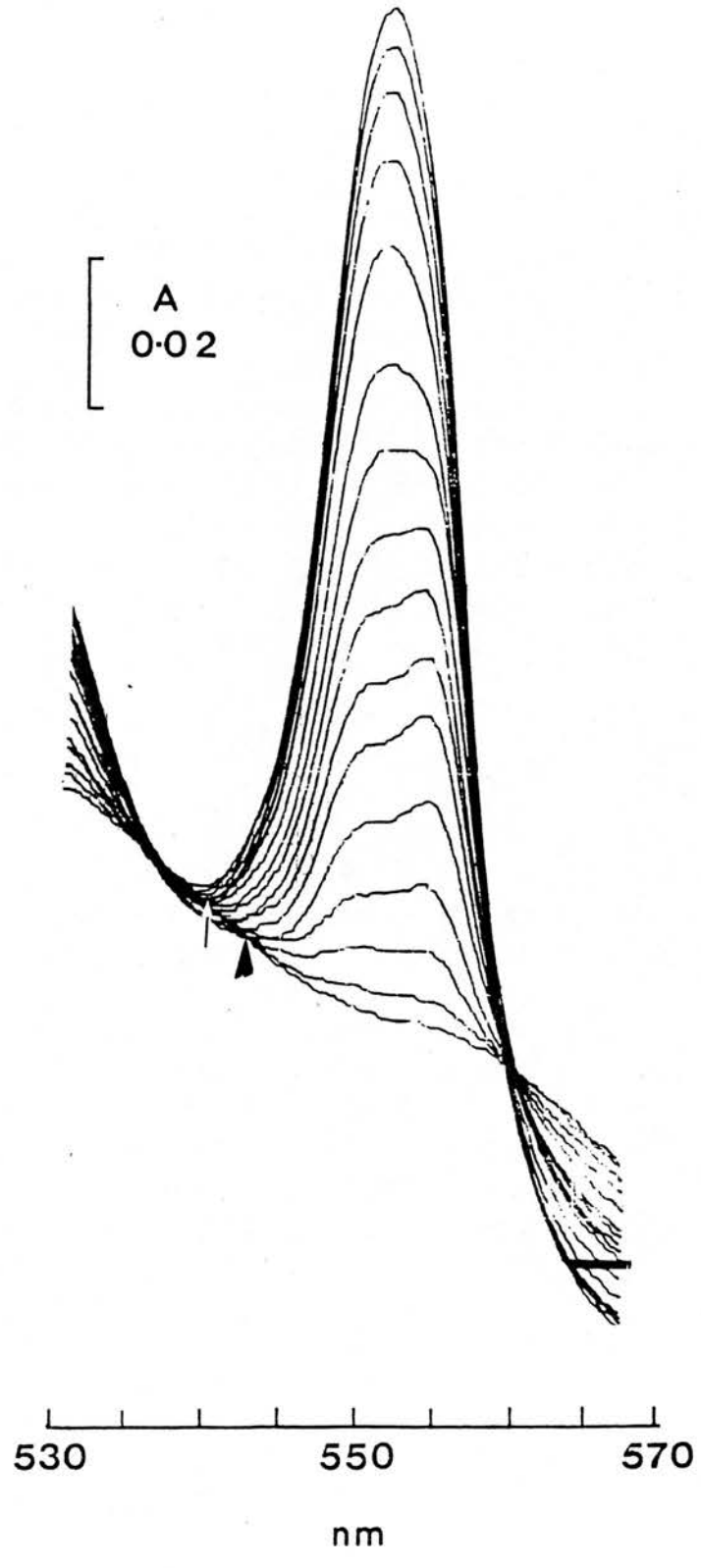
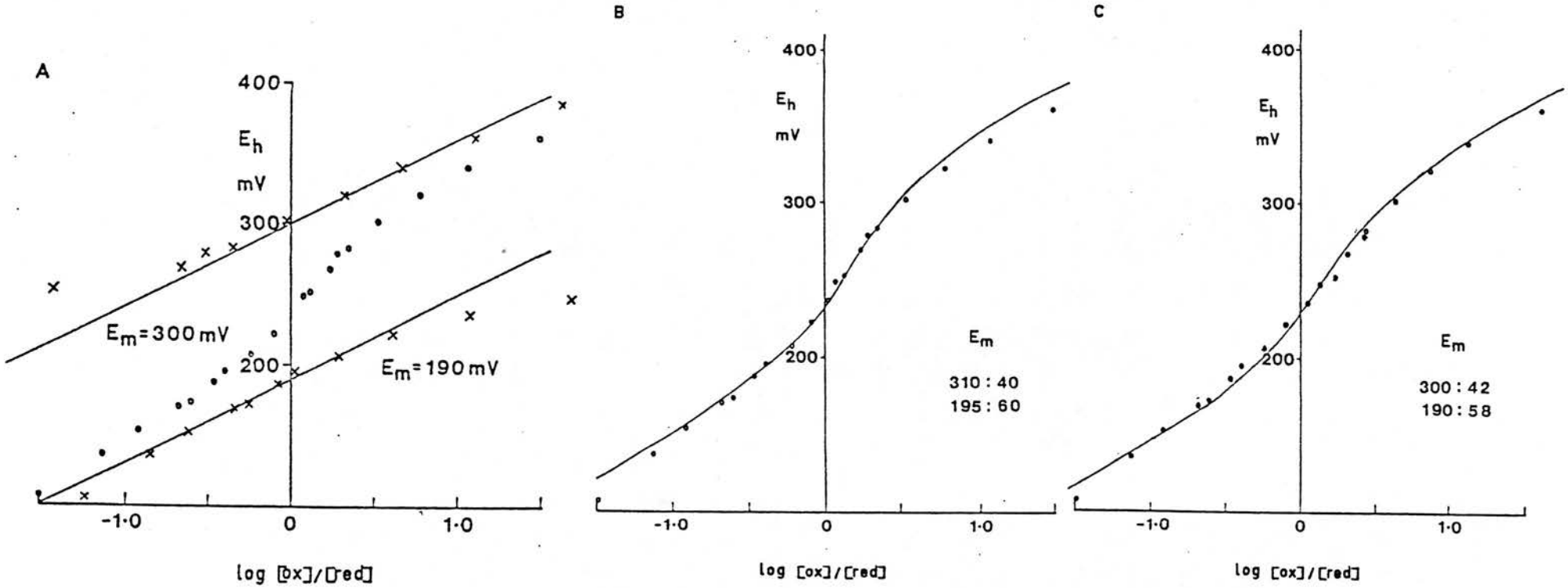

A
0·02
530
550
570
nm

Figure 42 : Nernst Plots for Cytochrome c_4

(a) Analysis of experimental points at 550nm. Open circles show the experimental points, crosses show points which were resolved arithmetically from the composite curve. The lines are slopes of 60mV.

(b) and (c) show the theoretical curves for analysis at the α-peak maximum and at 550nm respectively. Curves calculated from the disected components. For (b) and (c) the open circles and closed circles are oxidative and reductive titration results respectively.


A
400
E_h
mV
300
200
E_m=300 mV
E_m=190 mV
-1·0
0
1·0
log [ox]/[red]
B
400
E_h
mV
300
200
E_m
310 : 40
195 : 60
-1·0
0
1·0
log [ox]/[red]
C
400
E_h
mV
300
200
E_m
300 : 42
190 : 58
-1·0
0
1·0
log [ox]/[red]

abscissa) the 2 components can be separated to yield 2 plots of 60mV slope (see Figure 42 (a)). Figure 42 (b) and (c) show theoretical curves calculated from the two dissected components with particular absorbance contributions (for both α-peak and 550nm analyses respectfully).

These plots demonstrate that the redox titration of Pseudomonas stutzeri 224 cytochrome c_4 can be analysed in terms of two midpoint potentials and that analysis at the α-peak maximum or at 550nm have little effect on the calculated midpoint potentials.

CHAPTER VI : REDOX TITRATION OF CYTOCHROME c_4

Section III - Proteolytic Cleavage of Pseudomonas stutzeri 224 Cytochrome c_4 and Purification and Characterisation of the Fragments

A. Method of proteolysis

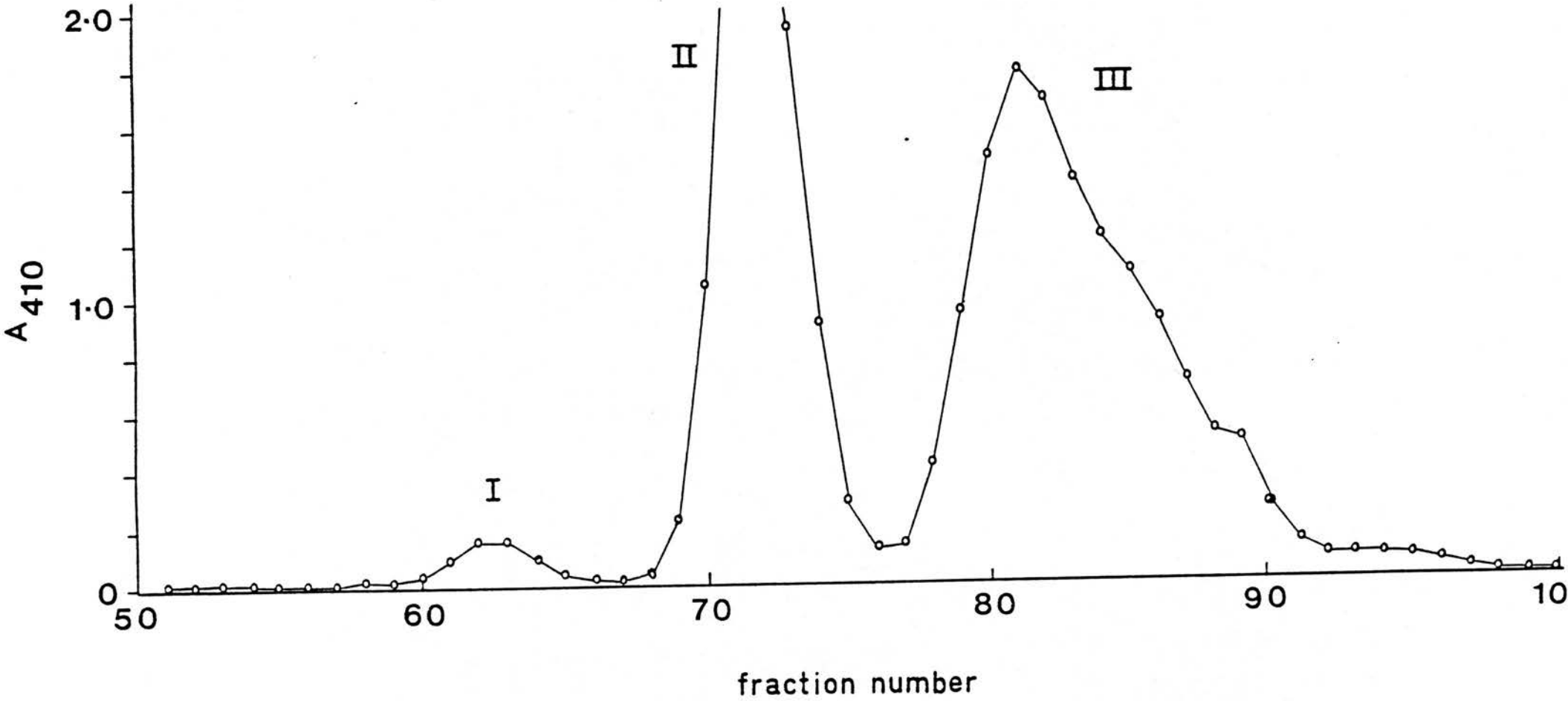
Cytochrome c_4 (purified from aerobic membranes) was subjected to proteolytic digestion by chymotrypsin (Sigma,bovine pancreas) (since in small scale digests chymotrypsin was shown to produce only two haem containing peptides with SDS-PAGE mobilities suggesting that they may be large enough to be the two domains required) using a protein to chymotrypsin ration of 10:1 (w/w) in 25mM sodium phosphate buffer pH 7. Incubation was carried out for 60 mins at 37°C. After incubation chymotrypsin was inhibited by addition of a 30 fold excess (mol/mol) of PMSF (Sigma).

B. Purification

The digest mixture was then passed through a column of Sephadex G-75 superfine (2x75 cm) equilibrated with 20mM Tris-HCl/100mM sodium chloride pH 8 (4°C). Fragments were pooled and concentrated by absorption on columns of DE-52 (1x2 cm) and stripped with 5mM Tris-HCl/500mM sodium chloride pH 8 (4°C) and finally desalted into 5mM Tris-HCl pH 8 (4°C) by passage through Sephadex G-25 superfine (2x8 cm).

Figure 43 shows the elution profile of the digest mixture from the Sephadex G-75 column. Samples from fractions across the profile were analysed by SDS-PAGE

Figure 43 : Elution of Digest Mixture from Sephadex G-75


2·0
1·0
0
A_{410}
I
II
III
50
60
70
80
90
10
fraction number

(not shown) which demonstrated that peak I contained undigested cytochrome c_4. Peaks II and III contained fragments of cytochrome c_4.

Table XIII shows the yields of the individual fragments. Starting with cytochrome c_4 (544 nmoles haem), and assuming 100% recovery, 272 nmoles of each fragment would have been expected. The yields of 140 and 66 nmoles for the large and small fragments respectively are 52% and 24% of the expected values. Some of the losses may be explained by losses during purification and also undigested cytochrome c_4 (value not determined but from the elution profile the amount compared to the individual fragments is very small).

Another possible loss may be due to progressive digestion of the individual fragments during the digestion period. It would appear from the results in Table XIII that the smaller fragment may be more susceptible to proteolytic digestion than the large fragment.

C. Properties of the large and small fragments

(i) Molecular weight

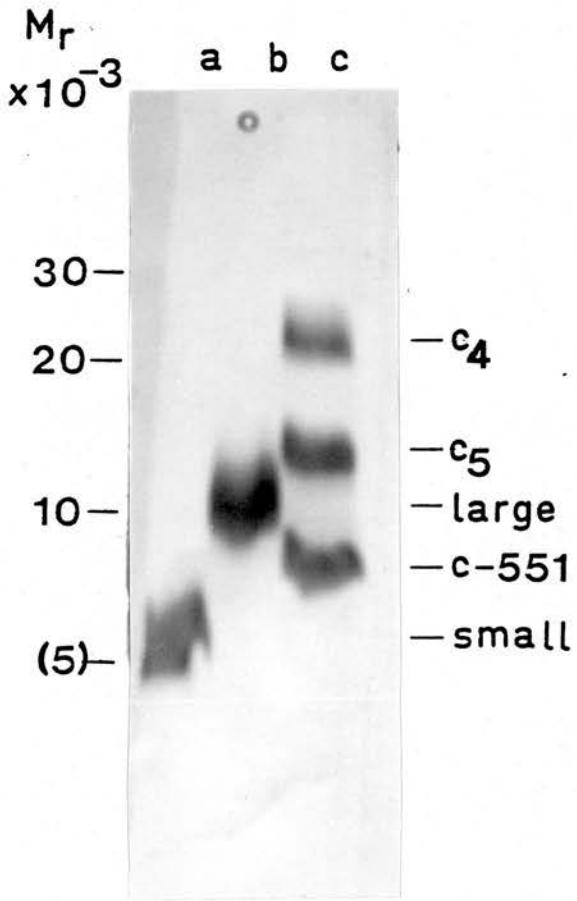
The molecular weight of each fragment was determined from their mobilities on a 20% acrylamide gel with 10% glycerol present (Cabral and Schatz, 1979) (see Figure 44). This gel system was used to obtain good resolution of low molecular weight proteins. Molecular weights of the fragments were calculated from a standard curve of log MW vs mobility relative to horse heart cytochrome c. The standard curve (not shown) was constructed from the

Table XIII : Purification of the Chymotryptic Fragments of Cytochrome c_4 : Yields of the Individual Fragments

	nmoles haem
Cytochrome c_4	544
Large fragment	140
Small fragment	66

Figure 44 : SDS-PAGE Analysis of the Large and Small Fragments of Cytochrome c_4

Gel prepared according to Cabral and Schatz (1979). Lanes (a) and (b) were the small and large fragments respectively. Lane (c) was loaded with purified cytochromes c_4, c_5 and c-551 (from Pseudomonas stutzeri 224). Molecular weights of the fragments and the M_r scale were calculated from the relative mobilities of a set of molecular weight marker proteins (not shown). Marker proteins (mol wt) used were ovalbumin (45000), yeast cytochrome c peroxidase (34000), horse heart cytochrome c (12000) Pseudomonas aeruginosa cytochrome c-551.


Mr
x10-3
a
b
c
30
20
10
(5)
c4
c5
large
c-551
small

protein standards (lane a). The molecular weights of the large and small fragments were 8900 and 5000 (this value is unreliable since it is outside the range of the molecular weight markers used). respectively. This gel also indicates that the 2 fragments are pure from other haem peptides.

(ii) Spectral properties of the large and small fragments

Figures 45 and 46 show spectra of the individual fragments and Table XIV summarises the spectral properties of the fragments and also those of cytochrome c_4 for comparison. The spectra indicate that there has been little disruption of the protein structure around the haem moeity in the large fragment as the presence of the 695nm band demonstrates a histidine-methionine ligation of the haem iron. However, the 695nm band is absent in the small fragment suggesting disruption of the protein structure around the haem although the low spin spectrum indicates that the haem still has 6 strong field co-ordinating ligands.

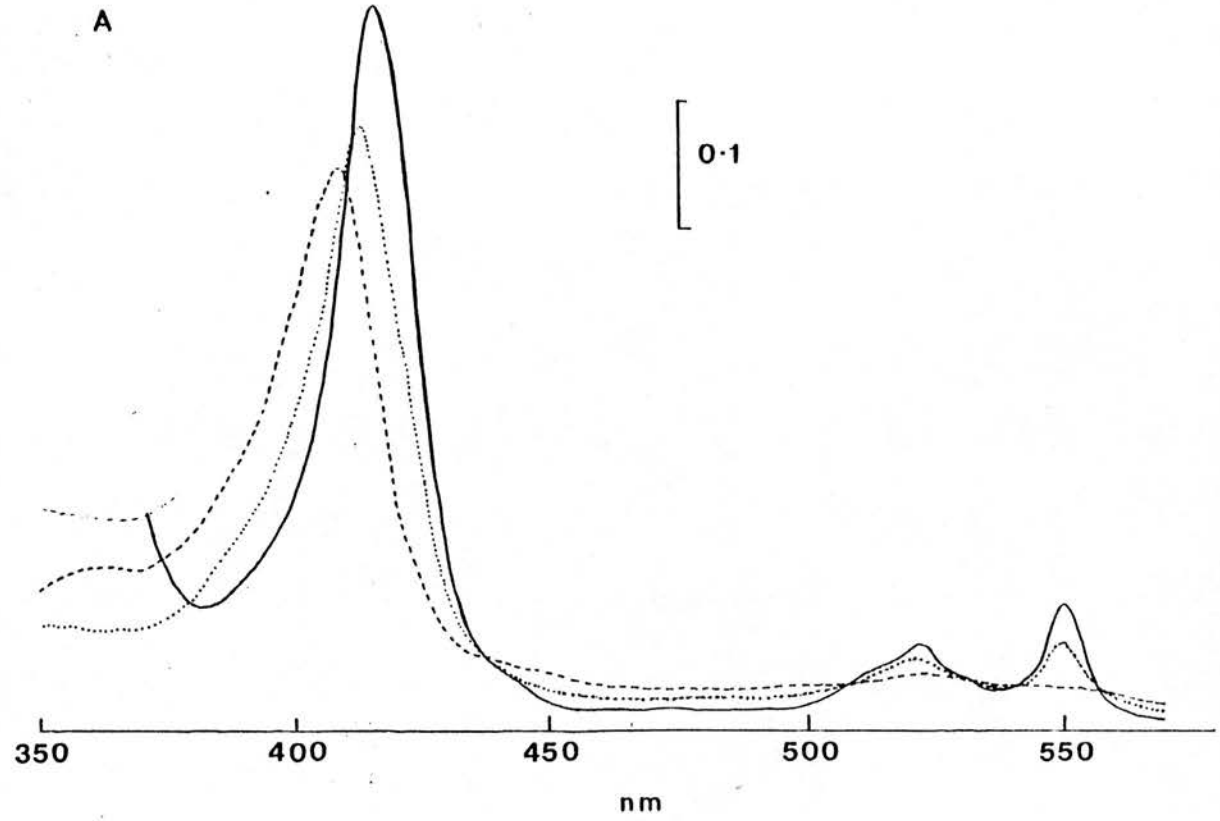
The α/β ratio of both fragments is higher than that of native cytochrome c_4.

(iii) Redox properties of the chymotryptic fragments

Figures 47 shows Nernst plots obtained from titration of the large and small fragments respectively. Neither midpoint potential corresponds to those of native cytochrome c_4. This result is not surprising since separating the domains may alter the influence the protein moeity has on the haems. The midpoint potential of -190mV for the small fragment may indicate the haem

Figure 45 : Spectra of the Large (A) and Small(B) Fragments of Cytochrome c_4 Recorded Between 350 and 600nm

Solid line shows the dithionite reduced spectrum, dotted line shows the ascorbate reduced spectrum and the dashed line shows the oxidised spectrum. The ascorbate reduced and oxidised spectra are identical in (B) and are shown as the mixed line.


A
0·1
350
400
450
500
550
nm

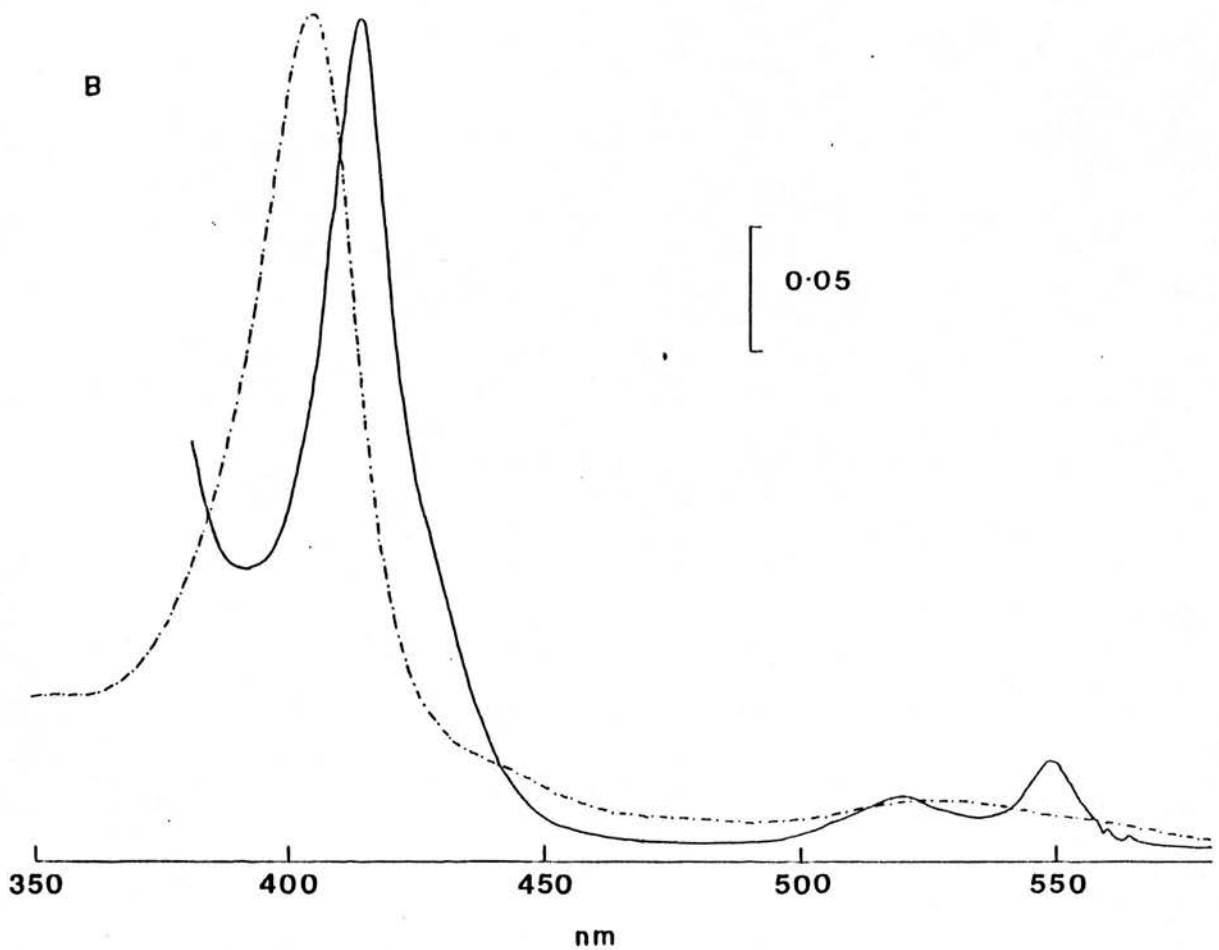

B
0·05
350
400
450
500
550
nm

Figure 46 : Spectra of the Large (A) and Small (B) Fragments Recorded Between 500 and 580nm

Solid line shows the dithionite reduced spectrum, the dotted line the ascorbate reduced spectrum and the dashed line the oxidised spectrum. The mixed line in (B) represents the identical ascorbate and oxidised spectra.

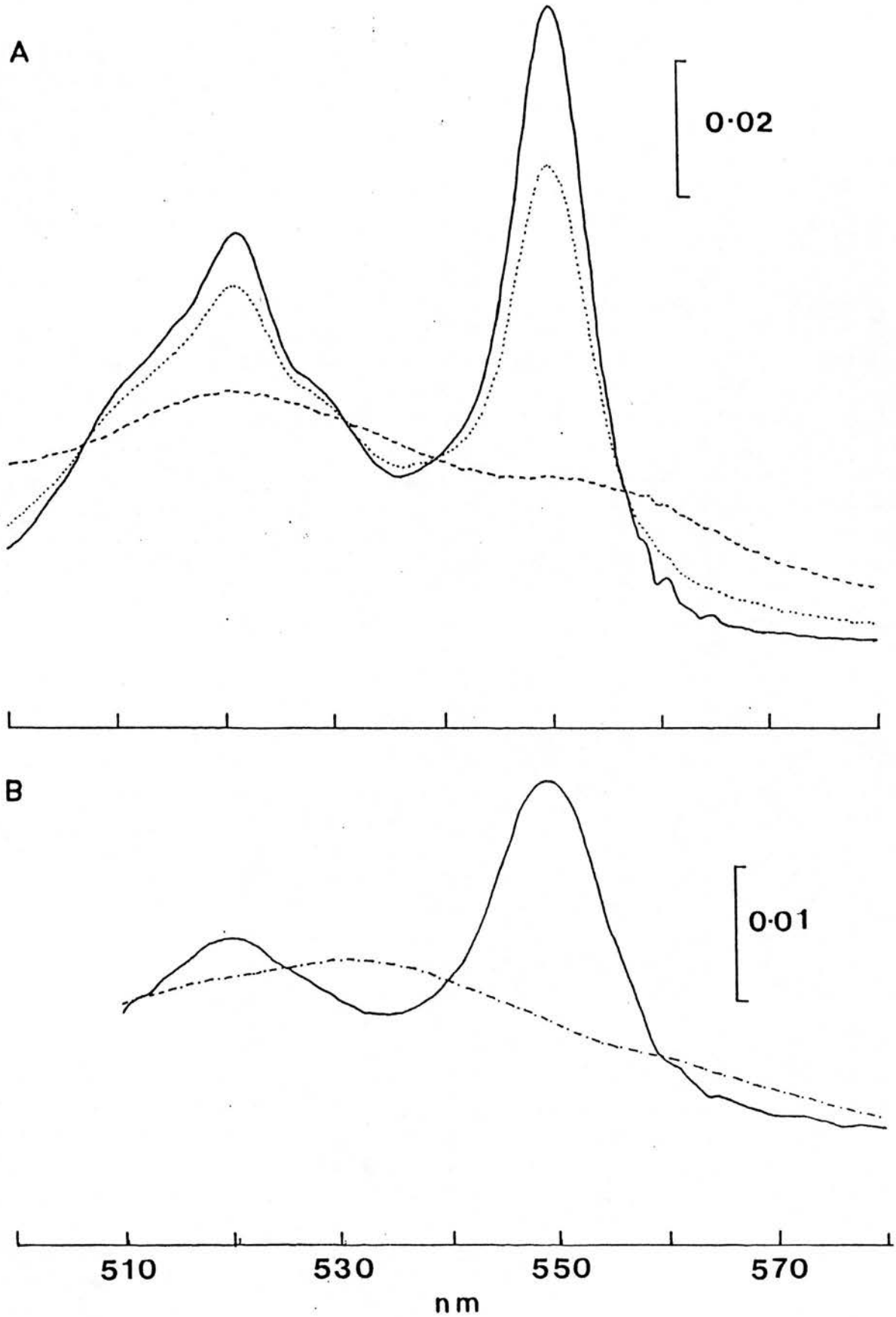

A
0·02
B
0·01
510
530
550
570
nm

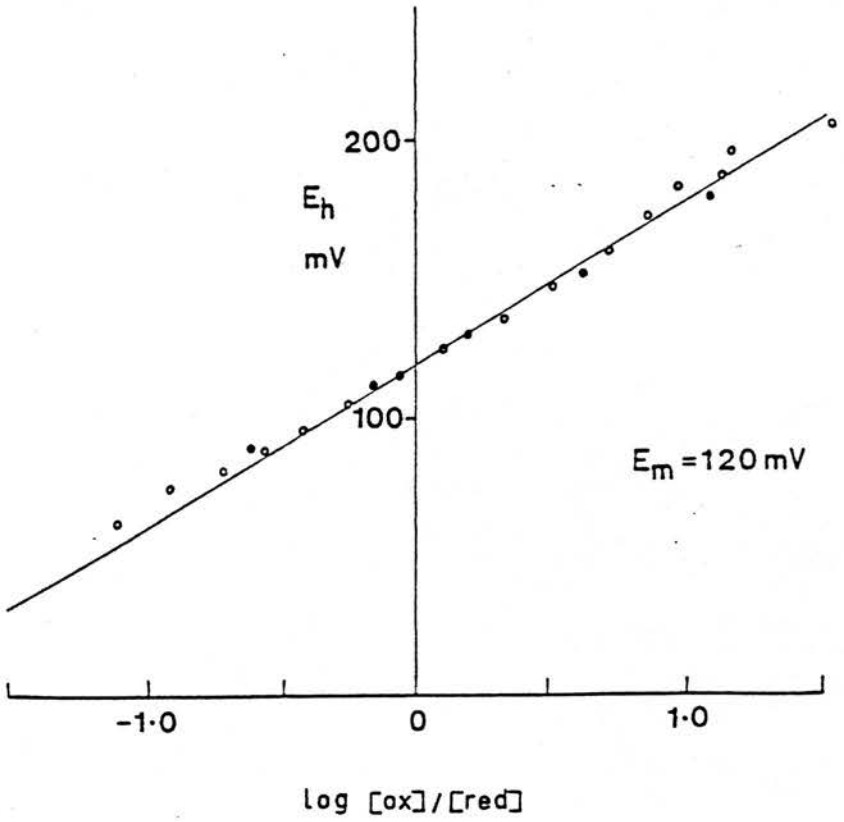
Table XIV : Spectral Properties of the Chymotryptic Fragments of Cytochrome c_4 and native Cytochrome c_4

	large fragment	small fragment	cytochrome c_4
α_{max}	550nm	549nm	550nm
β_{max}	521nm	520nm	552nm
γ_{max}(ox)	408nm	405nm	408nm
γ_{max}(red)	414nm	414nm	415nm
α/β ratio	1.57	1.46	1.21
695nm	yes	no	yes

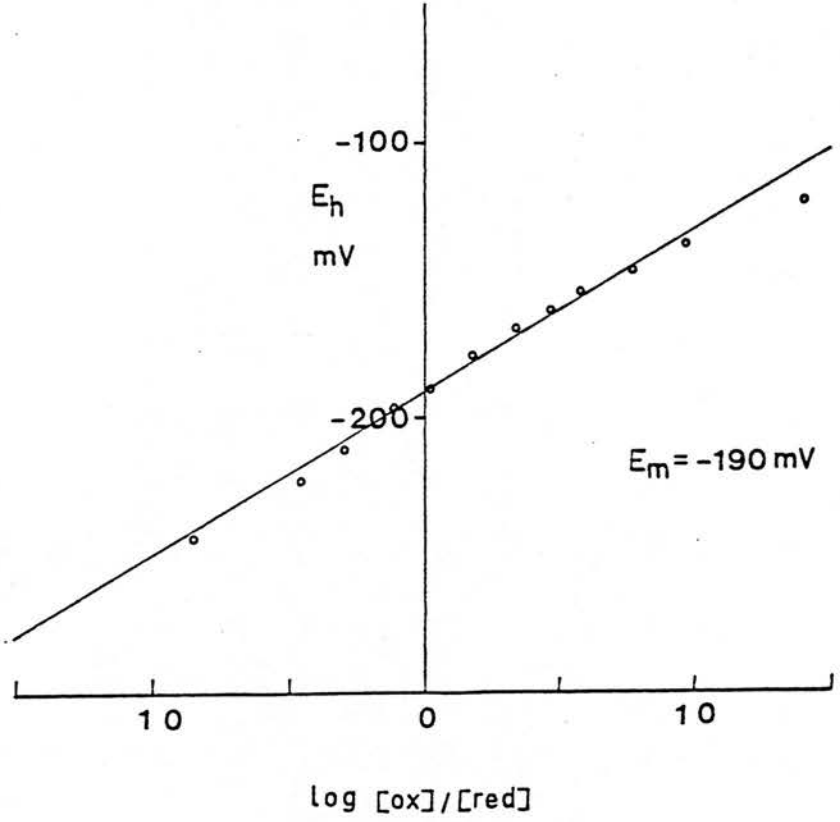
Figure 47 : Nernst Plots of the (A) Large and (B) Small Fragments of Cytochrome c_4

Open circles show points from the oxidative titration and closed circles the reductive titration. In the case of the small fragment it was only possible to collect data points for the oxidative titration.

A


200
100
E_h
mV
E_m = 120 mV
-1·0
0
1·0
log [ox]/[red]

B


-100
-200
E_h
mV
E_m = -190 mV
1 0
0
1 0
log [ox]/[red]

has been exposed (Harbury and Loach, 1959). The molecular weight of this fragment, as calculated from the amino acid analysis and from the SDS electrophoresis, indeed suggests that a portion of the domain must have been removed.

Examples of cytochromes, eg cytochrome c_3, are known which have such low redox potentials. It is possible that after proteolysis the protein folding around the haem has been altered leading to replacement of the methionine ligand and greater exposure of the haem.

(iv) Amino acid compositions of the two fragments

The amino acid compositions of the fragments are presented in Table XV. The molecular weight of each fragment was calculated from these analyses on the basis of 2 cysteines and 1 haem (assuming each fragment possesses 1 haem covalently attached to the polypeptide chain by 2 thioether linkages). The values of 10 236 and 6411 are in fairly good agreement with the values determined by SDS-PAGE. Since the oxidised holo-forms of the fragments were analysed by SDS-PAGE it may be that the molecular weight calculated by this method is an underestimate (see Chapter III Section B for discussion of effect of reduction state on mobility on SDS gels).

Table XVI shows the amino acid composition of the combined fragments, the native cytochrome c_4 composition and also the difference between the cytochrome c_4 and the sum of the 2 fragments. This difference will be called the "missing" chain (ie the chain lost during proteolytic

Table XV : Amino Acid Composition of the Large and Small Fragments of Cytochrome c_4

Residue	Large Fragment			Small Fragment		
	nmoles	residues (mol/mol haem)	(integral)	nmoles	residues (mol/mol haem)	(integral)
Asp	40.00	9.95	10	37.21	9.26	9
Thr	8.74	2.18	2	8.42	2.09	2
Ser	18.51	4.60	5	8.42	2.09	2
Glu	45.55	11.33	11	22.08	5.49	5
Pro	19.21	4.78	5	14.32	3.56	4
Gly	60.16	14.97	15	38.39	9.55	10
Ala	50.51	12.56	13	30.42	7.57	8
Val	20.32	5.05	5	10.58	2.63	3
Met	6.61	1.64	2	4.80	1.19	1
Ile	7.24	1.80	2	3.48	0.86	1
Leu	29.51	7.34	7	15.83	3.94	4
Tyr	16.92	4.21	4	2.04	0.51	1
Phe	4.52	1.12	1	3.53	0.88	1
His	6.63	1.65	2	8.80	2.19	2
Lys	24.28	6.04	6	8.50	2.11	2
Arg	7.34	1.83	2	2.61	0.65	1
MW			10236			6411

Table XVI : Amino Acid Composition of the Sum of the Amino Acid Compositions of the Fragments in Comparison with the Native Cytochrome c_4.

Column 3 is the Amino Acid Composition of the "missing" chain and the 4th Column is an Amino Acid Composition of a Fragment Identified from the C-terminus and Column 5 a Fragment Identified from the N-terminus of the C-terminal Domain of Cytochrome c_4 of Azotobacter vinelandii (see Fig 48)

Residue	Combined fragments	Cytochrome c_4	"missing" chain	Azotobacter "missing" chain	
				C	N
Asp	19	19	0	6	2
Thr	4	5	1	0	2
Ser	7	9	2	4	0
Glu	16	20	4	1	4
Pro	9	11	2	0	3
Gly	25	28	3	2	5
Ala	21	23	2	4	4
Val	8	8	0	0	2
Met	3	5	2	2	1
Ile	3	5	2	4	0
Leu	11	15	4	3	3
Tyr	5	5	0	1	1
Phe	2	4	2	0	0
His	4	4	0	1	0
Lys	8	11	3	2	2
Arg	3	6	3	1	1

cleavage). The final column represents the composition of a section of the Azotobacter vinelandii cytochrome c_4 (the source of these figures will be discussed below).

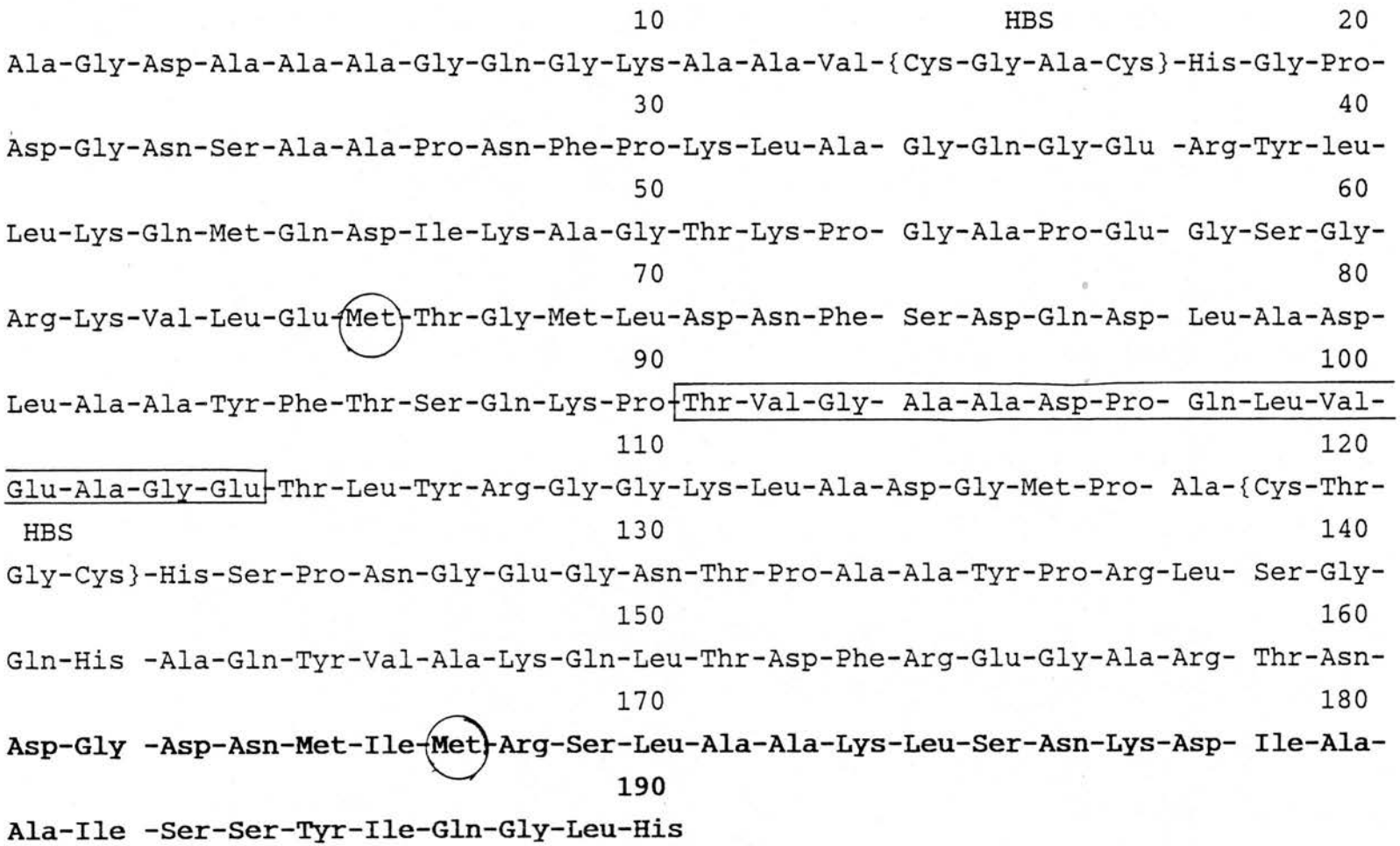
It would be interesting to look at the cytochrome c_4 sequence and attempt to identify a section of the polypeptide chain which corresponds to the amino acid composition of the "missing" chain. However the sequence of Pseudomonas stutzeri cytochrome c_4 is not known therefore the Azotobacter vinelandii cytochrome c_4 sequence has been used. The Azotobacter vinelandii sequence was selected since the Cornish-Bowden analysis suggests that it resembles the Pseudomonas stutzeri cytochrome c_4 sequence more closely than the others (calculated difference of 21% obtained).

Figure 48 shows the amino acid sequence of the Azotobacter vinelandii cytochrome c_4 (Ambler, 1984) with the boxed in region of the chain showing the position of the connecting loop. N-terminal analysis of native Pseudomonas stutzeri 224 cytochrome c_4 and of the large fragment revealed that they have the same N-terminal amino acid (alanine) suggesting that the large fragment consists of the domain from the N-terminus to the connecting loop. The small fragment has a ragged N-terminus (identification of the amino acids not attempted) suggesting it has been produced by the effect of chymotrypsin.

In addition the position of the 30 residue portion is located in the small fragment and must include two methionine residues. To achieve this a 30 amino acid

Figure 48 : Amino Acid Sequence of Azotobacter vinelandii Cytochrome c_4 (Ambler et al, 1984)

Circled methionine residues (positions 66 and 167) are the 6th coordinating ligands for the 2 haems. The 2 haem binding sites (HBS) are marked, 1 for each domain. The histidine residues at positions 18 and 123 are the 5th coordinating ligands for the 2 haems. The boxed region of the chain is the proposed position of the polypeptide chain connecting the 2 domains and the amino acids in bold type represent the proposed "missing" chain. The amino acid composition of this "missing" chain is shown in column 4 of Table XVI.


10
HBS
20
Ala-Gly-Asp-Ala-Ala-Ala-Gly-Gln-Gly-Lys-Ala-Ala-Val-{Cys-Gly-Ala-Cys}-His-Gly-Pro-
30
40
Asp-Gly-Asn-Ser-Ala-Ala-Pro-Asn-Phe-Pro-Lys-Leu-Ala- Gly-Gln-Gly-Glu -Arg-Tyr-leu-
50
60
Leu-Lys-Gln-Met-Gln-Asp-Ile-Lys-Ala-Gly-Thr-Lys-Pro- Gly-Ala-Pro-Glu- Gly-Ser-Gly-
70
80
Arg-Lys-Val-Leu-Glu-Met-Thr-Gly-Met-Leu-Asp-Asn-Phe- Ser-Asp-Gln-Asp- Leu-Ala-Asp-
90
100
Leu-Ala-Ala-Tyr-Phe-Thr-Ser-Gln-Lys-Pro-Thr-Val-Gly- Ala-Ala-Asp-Pro- Gln-Leu-Val-
110
120
Glu-Ala-Gly-Glu-Thr-Leu-Tyr-Arg-Gly-Gly-Lys-Leu-Ala-Asp-Gly-Met-Pro- Ala-{Cys-Thr-
HBS
130
140
Gly-Cys}-His-Ser-Pro-Asn-Gly-Glu-Gly-Asn-Thr-Pro-Ala-Ala-Tyr-Pro-Arg-Leu- Ser-Gly-
150
160
Gln-His -Ala-Gln-Tyr-Val-Ala-Lys-Gln-Leu-Thr-Asp-Phe-Arg-Glu-Gly-Ala-Arg- Thr-Asn-
170
180
Asp-Gly -Asp-Asn-Met-Ile-Met-Arg-Ser-Leu-Ala-Ala-Lys-Leu-Ser-Asn-Lys-Asp- Ile-Ala-
190
Ala-Ile -Ser-Ser-Tyr-Ile-Gln-Gly-Leu-His

portion from the C-terminus can be removed, ie back to residue 161. However, the amino acid composition of this fragment does not resemble that of Pseudomonas stutzeri cytochrome c_4.

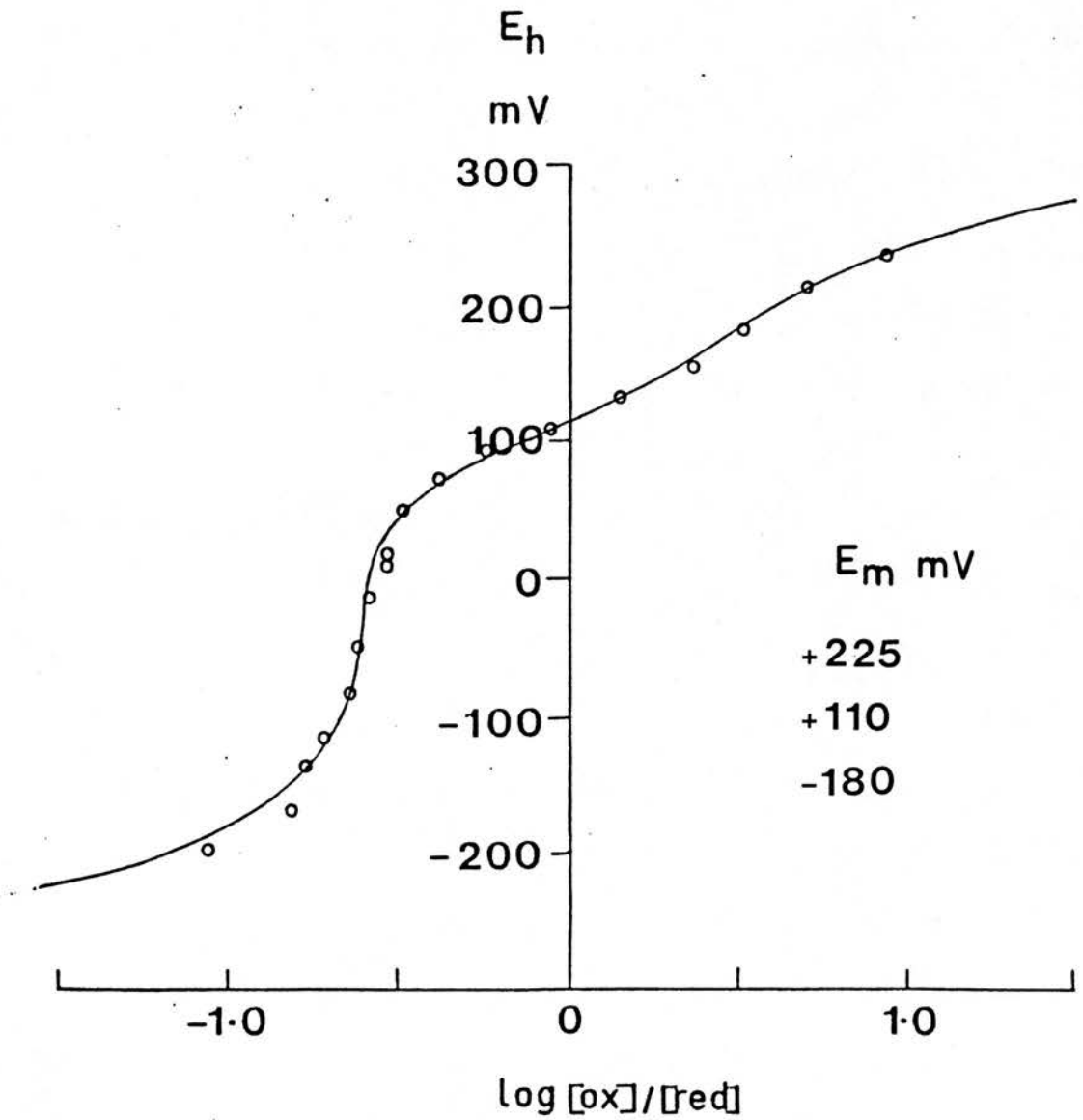
The removal of the 2 methionine reidues indicated is supported by the spectrum of the smaller fragment ie the 695nm band is absent, indicative of the loss of methionine as the 6th coordinating ligand. The methionine at position 66 is the 6th coordinating ligand for the haem iron.

In addition to the 30 amino acid portion being located at the C-terminal end of the small fragment a 30 amino acid portion from the N-terminal end of the small fragment should also be considered, eg from amino acid 88 to 117. This would remove one methionine residue and perturb the structure sufficiently for the methionine coordinated to the haem to be replaced by ,eg, a lysine. Of course the 30 amino acid residues may be removed from both the N- and C-terminus of the small fragment. It is difficult to identify a sequence from the Azotobacter sequence using an amino acid composition from a Pseudomonad.

D. Redox titration of an unfractionated mixture of proteolytically cleaved cytochrome c_4

The conditions for the chymotryptic digestion of cytochrome c_4 were the same as for the purification

Figure 49 : Redox Titration of the Unfractionated Chymotryptic Digest of Cytochrome c_4


E_h
mV
300
200
100
0
-100
-200
-1·0
0
1·0
log [ox]/[red]
E_m mV
+225
+110
-180

experiments except that 50nmoles of cytochrome was digested and no fractionation of the mixture took place. The mixture was titrated immediately after the addition of PMSF.

SDS-PAGE analysis of the digest mixture (not shown) demonstrated the presence of 2 fragments which run with the same mobility as the purified fragments. Figure 49 shows a Nernst plot of such a mixture which can be analysed in terms of three components, two of which correspond to the midpoint potentials of the purified fragments, ie the +110mV potential corresponds to that of the purified large fragment and the -180mV potential corresponds to that of the purified small fragment. In addition to the +110 and -180mV potentials a potential of +225mV was observed. Since the percentage contribution of the +225mV potential is only approx. 5% it is impossible to say whether this region constitutes a single redox potential of +225mV, which may indicate an intact, proteolytically separated domain, or whether a further two components could be fitted with potentials corresponding to those of native cytochrome c_4 (ie some undigested cytochrome c_4 may still be present in the mixture).

The +110mV fragment was shown to correspond to the haem near the N-terminus of cytochrome c_4 but it is not known whether this +110mV fragment corresponds to the higher or lower potential observed in native cytochrome c_4. However, without an intact "small fragment" it is impossible to determine if the haems are identical in the

oxidised form or are intrinsically differnt and if intrinsically different does the small fragment have a higher or lower potential than the large fragment.

If the +225mV potential corresponds to an intact "small fragment" this would support the intrinsically different model. It could be imagined that even although this +225mV domain still retains full structure around the haem, the polypeptide chain has been nicked and a 30 amino acid portion of the chain would be free to dissociate from the rest of the domain giving rise to the -190mV fragment observed in both the mixture and the purification experiments. If this was the case then passage of the mixture through Sephadex G-75 may ensure complete dissociation so that a +225mV fragment was not obseved during the purification (gel electrophoresis in SDS would also of course dissociate the peptide from the rest of the domain thus it would be seen as the small fragment and not as a higher molecular weight band).

CHAPTER VII : PARTIAL CHARACTERISATION OF THE 30K PROTEIN

A c-type cytochrome of approx. molecular weight 30 000 is greatly induced when Pseudomonas stutzeri 224 is grown in nitrate medium suggesting that it plays some role in the denitrification process. Nothing is known of the function of this cytochrome. Villalain et al (1981) have reported the purification and characterisation of a 28K c-type cytochrome from Pseudomonas perfectomarinus (which may be a close relative of Pseudomonas stutzeri) and noted that it possesses peroxidase activity. Liu et al (1981) have also noted the presence of a similar protein in Pseudomonas stutzeri 224

A. Purification of the 30K protein

Pseudomonas stutzeri 224 were grown anaerobically with nitrate as terminal acceptor (as described earlier). Cells were suspended in 4 Vols 20mM sodium phosphate buffer pH 7 containing 2mg DNase and 1mM PMSF. Cells were broken by passage through a French pressure cell. Membranes were pelleted by centrifugation (100 000 x g) for 1 hr. The supernatant (which contains the 30K) was removed and stored on ice. The membranes were resuspended in 20mM sodium phosphate buffer, pH 7 and centrifuged as above. The supernatant was removed and added to the first. The combined supernatants were desalted into 5mM Tris-HCl pH 8 ($4^{o}C$) by passage through Sephadex G-25 (coarse) and then absorbed onto DE52 (5 x 10cm). The column was developed with a sodium chloride gradient, 0-150mM NaCl in 5mM Tris-HCl, pH 8 ($4^{o}C$).

Fractions containing 30K were identified by SDS-PAGE stained for haem. These fractions were pooled, concentrated on DE52, desalted into 5mM Tris-HCl, pH 8 (4°C) and applied to a column of Sephadex G-75 (superfine) equilibrated with 20mM Tris-HCl/100mM NaCl pH 8 (4°C). The 30K fractions were pooled, absorbed onto DE52 (2 x 5cm) and developed with a gradient of NaCl, 50-200mM, in 20mM Tris-HCl pH 8 (4°C).

After this stage the 30K band is free from other haem proteins but still contaminated with non-haem proteins of around 25-35K. The sample was absorbed onto hydroxylapatite (2 x 8cm) at room temperature and the column then developed with a sodium phosphate, pH 7, gradient (5-200mM). Fractions were read at 410nm and 280nm and samples from fractions across the main 410nm were analysed by SDS-PAGE stained for haem and protein. This gel indicated the presence of only one haem stained band but two on the protein stain which run very close together.

Even although the 30K band is not pure from other proteins, the absence of other haem proteins enables partial characterisation to be carried out.

B. Characteristics of the 30K band

(i) Spectral properties

Figures 50 and 51 show spectra of the 30K band, the results of which are summarised in Table XVIII . Figure 50 shows the spectrum over the range 750-350nm. Three spectra were recorded - ie oxidised, ascorbate reduced and dithionite reduced. Addition of ascorbate results

Figure 50 : Spectrum of the 30K Protein

The oxidative spectrum (broken line), ascorbate reduced spectrum (dotted line) and dithionite reduced spectrum were recorded between 350 and 580nm. The mixed line shows that the oxidised and ascorbate reduced spectra are identical.

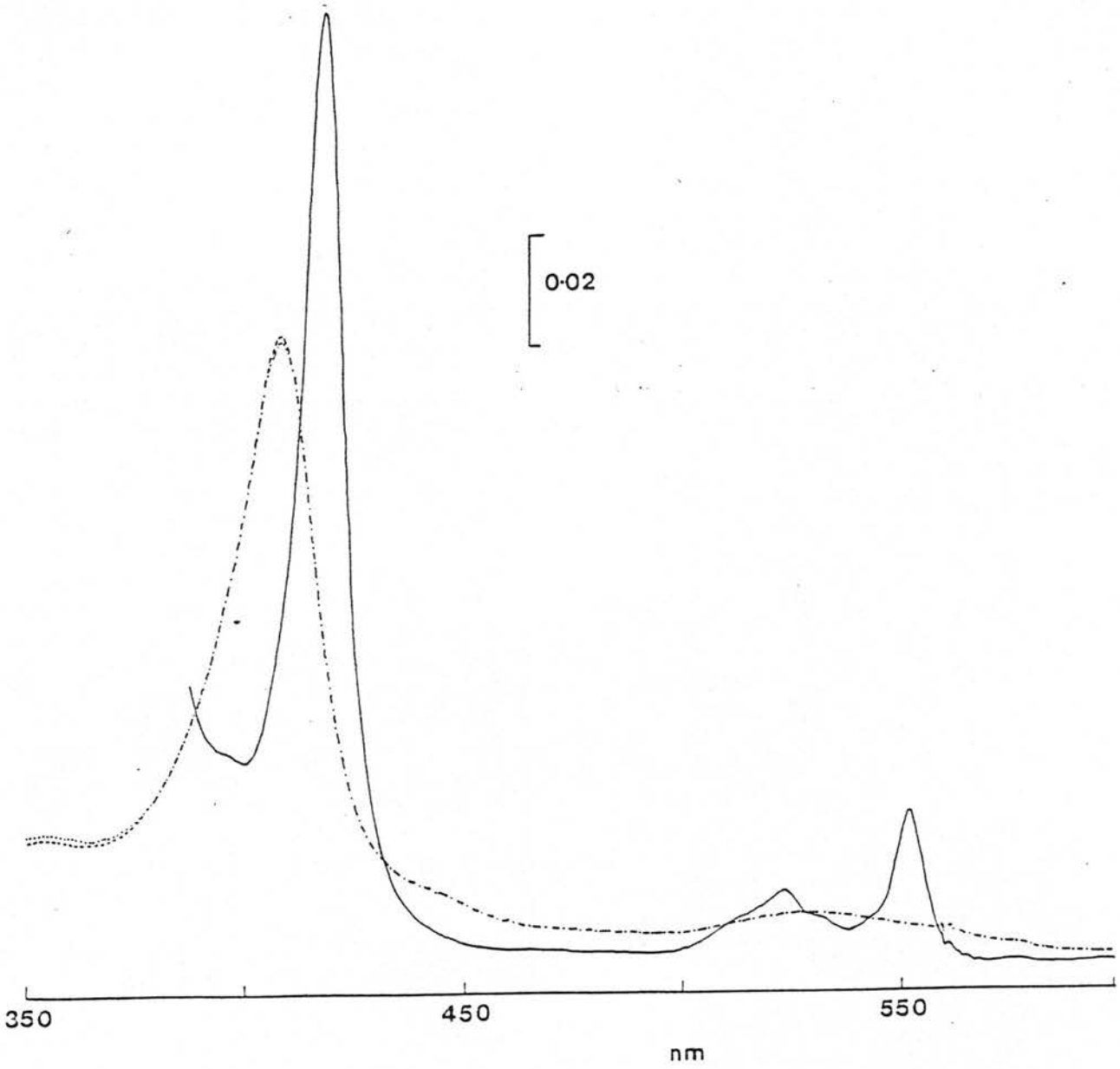

0·02
350
450
550
nm

Figure 51 : Spectrum of the 30K Protein

The oxidised (broken line), ascorbate reduced (dotted line) and dithionite reduced (solid line) spectra were recorded between 510 and 580nm (mixed line shows that the ascorbate reduced and oxidised spectra are the same).

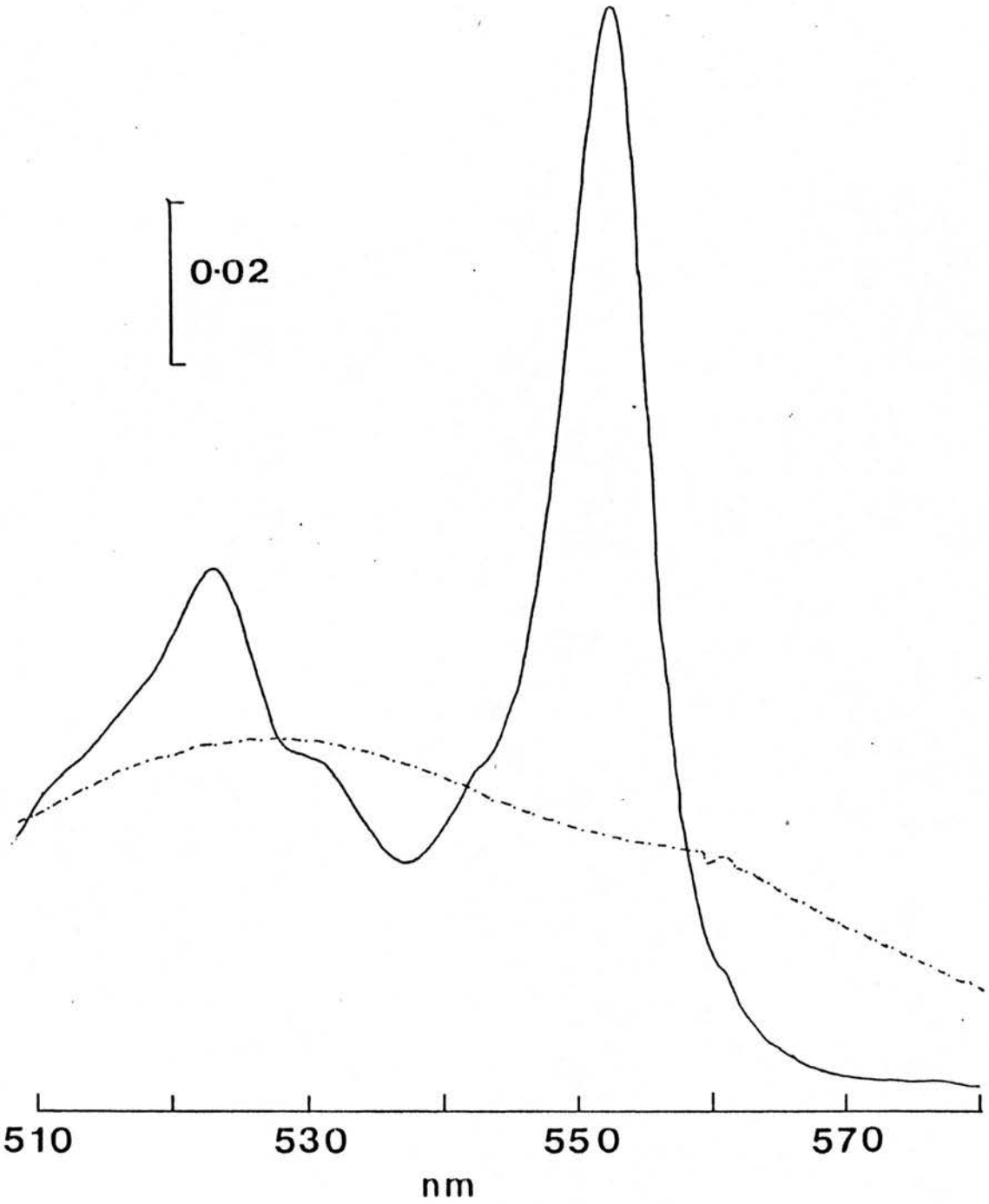

0·02
510
530
550
570
nm

Table XVIII : Summary of spectral properties of 30K band compared to the P. perfectomarinus protein

	P stut.	P. perfecto (Liu et al 1981)
α_{max}	551.5nm	551.7nm
β_{max}	522.5nm	523nm
Soret (ox)	408nm	408.5nm
Soret (red)	418nm	418nm
α/β ratio	1.89	2.10
695 nm band	No	No
reducible by ascorbate	No	1/2 reduction

in no reduction at all since the ascorbate spectrum is virtually identical to that of the oxidised - ie for both oxidised and ascorbate spectra the Soret peak maximum is 418nm. Dithionite produces reduction of the cytochrome shifting the Soret peak maximum to 408nm and also produces the α-and β-peaks which are characteristic of a reduced cytochrome.

The α- and β-peak regions of the spectrum were expanded and are shown in Figure 51. This demonstrates an α-peak of 550nm and a β-peak of 522.5 nm with a shoulder on the β-peak at approx 530 nm

The spectrum in Figure 52 was produced from an oxidised sample of the 30K protein and shows no evidence of a 695 nm band indicating that the haems do not have histidine-methionine ligation. However, no high spin bands were seen suggesting some form of low spin co-ordination of the haems.

(ii) Redox properties of the 30K band

Figure 53 shows a Nernst plot for the titration of the 30K band which has been analysed in terms of two components. Redox potentials of +20 and -190mV were obtained. The cytochrome c-552 from Pseudomonas perfectomarinus yielded midpoint potentials of +174 and -180mV.

(iii) Peroxidase activity of the 30K band

The peroxidase activity of the 30K was tested using reduced cytochromes c_4, c_5 and C-551 (purified from Pseudomonas stutzeri 224).

Figure 52 : Investigation of the 695nm Band of the 30K Protein

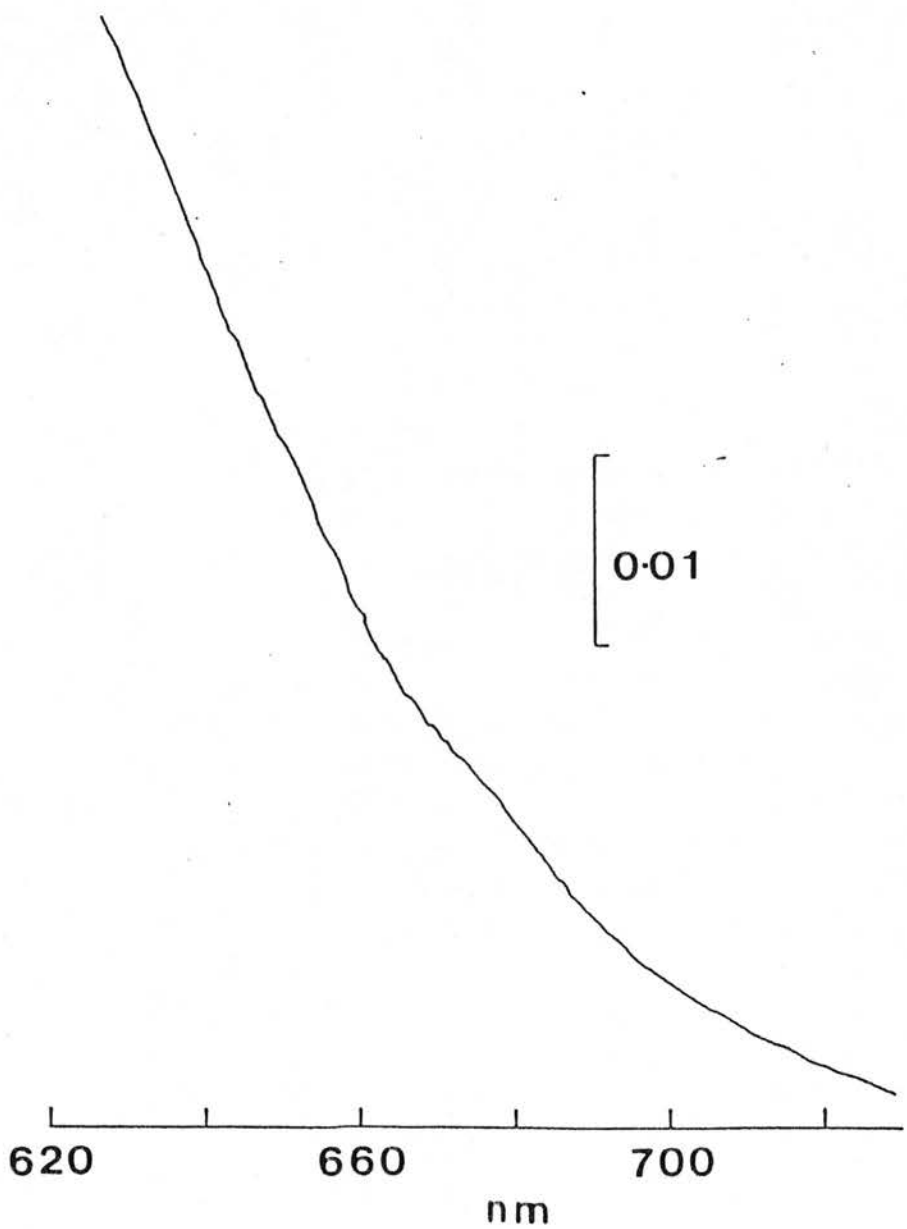
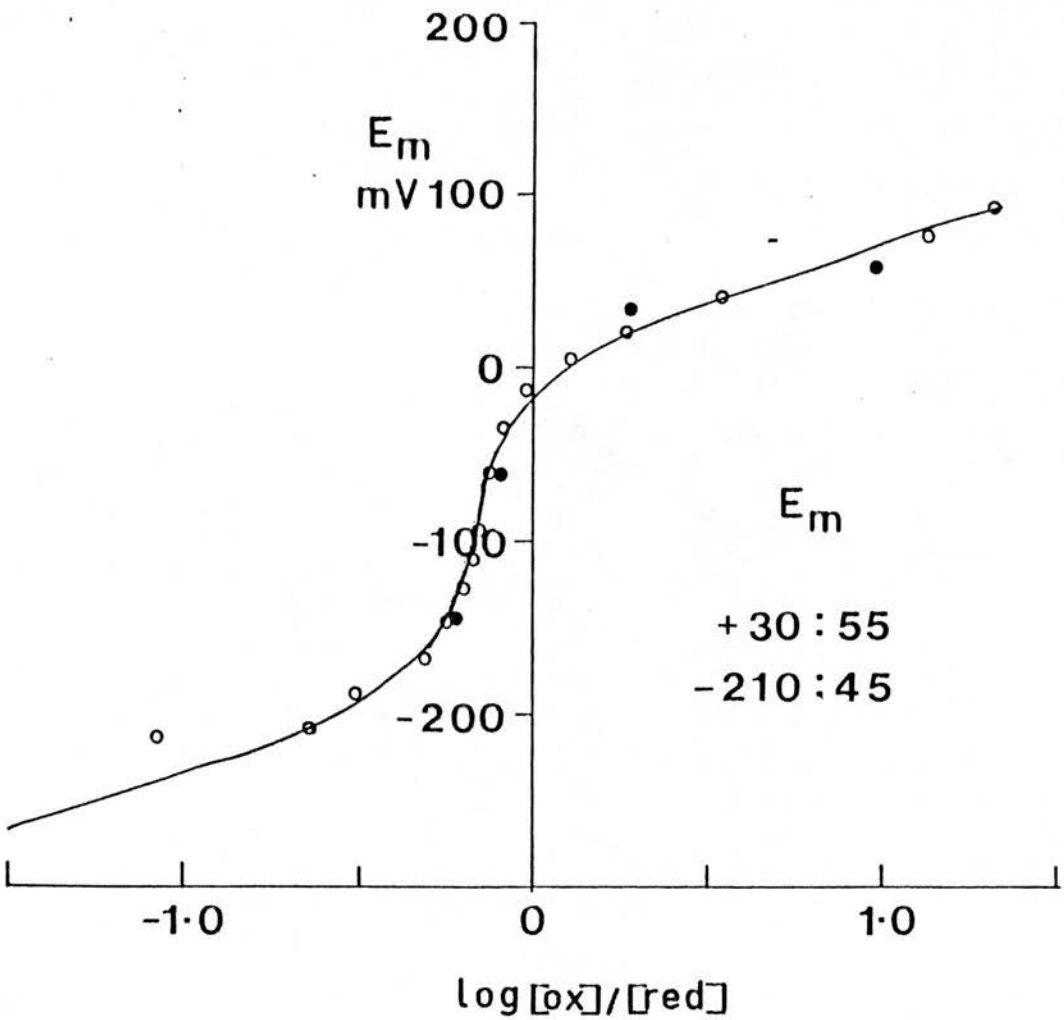

0·01
620
660
700
nm

Figure 53 : Redox Titration of the 30K Protein

Oxidative (open circles) and reductive (closed circles) titrations were carried out.


200
E_m
mV 100
0
-100
-200
E_m
+30 : 55
-210 : 45
-1·0
0
1·0
log [ox]/[red]

(a) Method : The donor cytochrome was approx. 5μM in 20mM sodium phosphate, pH 7 in a 3ml cuvette. Anaerobiosis was achieved by bubbling with argon for 15 mins after which the cytochrome was reduced by titrating with sodium dithionite (approx 8 mg/ml in 20mM phosphate). Absorbance changes were monitored at 551nm, 550nm and 554nm for cytochromes C-551, c_4 and c_5 respectively. The absorbance was monitored for about 1 min to demonstrate that reoxidation was not taking place. Hydrogen peroxide was added to a final concentration of 17.3mM and again any oxidation of the cytochromes was monitored (approx 1 min). A solution of 30K was added (final concentration μM) and the change in absorbance monitored.

(b) Results : Figure 54 shows the traces obtained from such experiments. For the three donor cytochromes no peroxidase activity was noted for the 30K band. This agrees with the results of Villalain et al (1984) who demonstrated that the cytochrome C-552 from Pseudomonas perfectomarinus has no peroxidase activity.

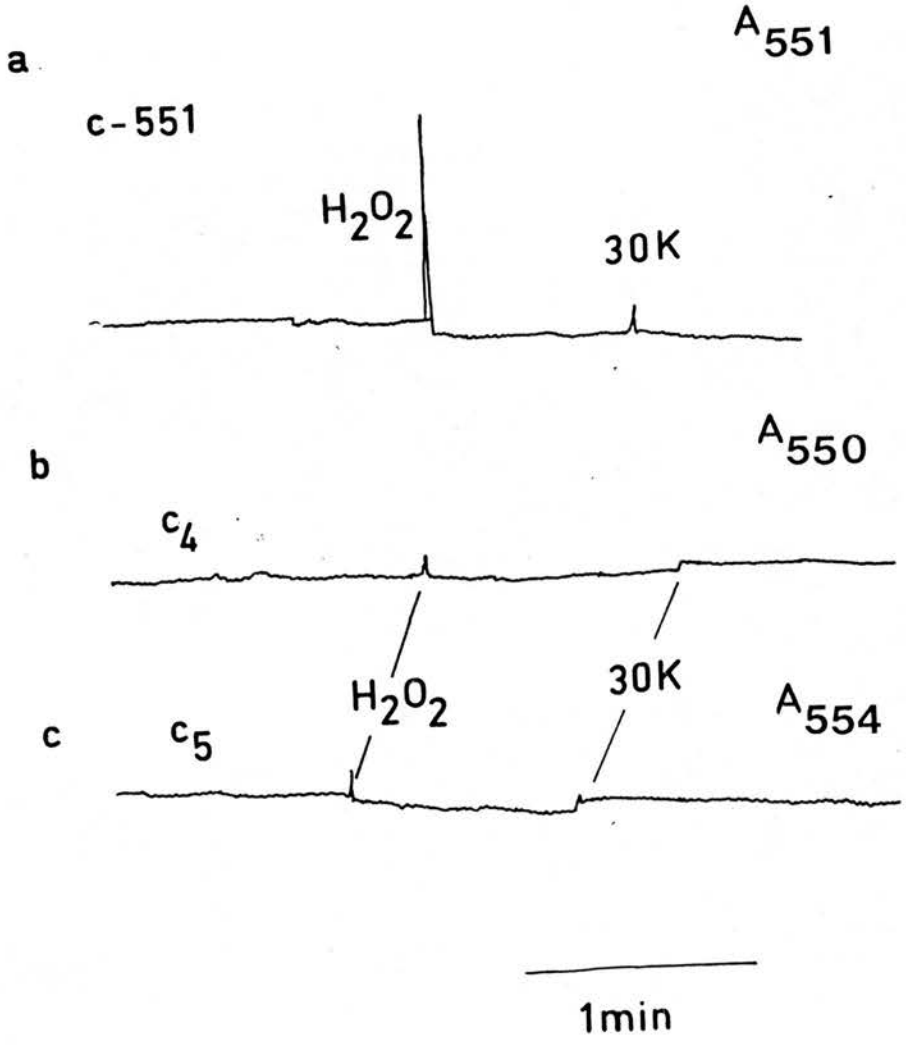
C Pyridine haemochrome of the 30K band

This was carried out as described in Methods. The α-peak maximum for the pyridine was 549nm and the millimolar extinction coefficient calculated for the native α-peak maximum (551.5nm) was 34.4.

D. Molecular weight

Figure 54 : Peroxidase Activity of the 30K Protein

Cytochrome c-551 (a), cytochrome c_4 (b) and cytochrome c_5 were investigated as possible electron donors to the 30K protein.


a
A551
c-551
H2O2
30K
b
A550
c4
H2O2
30K
A554
c
c5
1min

The molecular weight was calculated from SDS acrylamide gels from the mobility relative to cytochrome c_4. A plot of mobility of molecular weight standards relative to c_4 against log molecular weight was constructed. The molecular weight of the 30K band was calculated to be 31400.

CHAPTER VIII : DISCUSSION

Section I - Induction and Location of Cytochrome c_4

A. Induction

During the course of this study the effects of growth conditions on the levels of cytochrome c_4 in the bacterium Pseudomonas stutzeri 224 were examined. Three metdods were employed to quantify cytochrome c_4, namely (1) quantitation by purification, (2) quantitation from haem stained gels and (3) quantitation from Western blots. Each method has advantantages and disadvantages, which were discussed in Chapter IV.

For purposes of the following discussion the results of the quantitation by purification experiments will be used and discussed in relation to cytochrome c_4 levels in other bacterial species (ie Pseudomonas aeruginosa (Pettigrew and Brown, 1988) and Pseudomonas stutzeri (van Niel Strain) (kodama, 1970).

The results of the quantitation by purification of cytochrome c_4 are presented diagramatically in Figure 27 and numerically in Table XVIII(A). These results demonstratetwo points : (1) The overall level of cytochrome c_4 was virtually unaffected by altering the conditions of growth from aerobic to nitrate and (2) the distribution of cytochrome c_4 between membrane and soluble fractions is dependent on growth conditions.

(1) The overall level of cytochrome c4 is unchanged: In addition to the results showing that the

Table XVIII : (A) Summary of the Results of Cytochrome c_4 Quantitation by the Purification Method and Comparison with (B) the Results of Kodama (1970) for Bound Cytochrome c-552 from Pseudomonas stutzeri van Niel strain

(A)

	Membrane (nmole/100g)	Soluble (nmole/100g)	total
Aerobic	410	71	481
Nitrate	309	161	470

(B)

	Bound cytochrome c-552 (nmoles haem/mg cell protein)
High aeration	0.36[a]
Anaerobic plus nitrate	0.74[b]
Anaerobic plus nitrite	1.36[a]

(a) Cells harvested at end of growth

(b) Cells harvested at end of 1st logarithmic phase of anaerobic, biphasic, growth.

level of cytochrome c_4 remains virtually unchanged between aerobic and nitrate growth in Pseudomonas stutzeri 224 Pettigrew and Brown (1988) have studied the effects of growth conditions on cytochrome c_4 levels in Pseudomonas aeruginosa where slightly more cytochrome c_4 was found in aerobically grown cells.

The results from both Pseudomonas stutzeri 224 and Psedomonas aeruginosa and the presence of cytochrome c_4 in the strict aerobe Azotobacter vinelandii suggests that cytochrome c_4 is involved in an aerobic process rather than playing a unique role in denitrification. The involvement of cytochrome c_4 in aerobic processes, and in particular the involvement with the terminal oxidase cytochrome o, will be discussed more fully in Section II of this chapter.

(2) The distibution of cytochrome c_4 between membrane and soluble fractions is dependent on growth conditions: In the case of Pseudomonas stutzeri 224 85% of the cytochrome c_4 is membrane bound in aerobically grown cells while only 65% of the cytochrome c_4 is membrane bound in nitrate grown cells. A similar, but much less pronounced effect was observed in Pseudomonas aeruginosa where no soluble cytochrome c_4 was detected in aerobic cells but small quantities (approx. 3%) of the cytochrome c_4 was found in the soluble fraction of nitrate grown cells (Pettigrew and Brown, 1988).

It may be that a relatively constant amount of cytochrome c_4 is synthesised (as indicated by the total cytochrome c_4 content of aerobic and nitrate grown

cells) regardless of the growth conditions but the amount of cytochrome c_4 bound to the membrane is dependent on the number of sites for binding cytochrome c_4 (eg the reductase and oxidase). Yang et al (1979) proposed that cytochrome c_4 was an integral part of the terminal oxidase cytochrome o. It is conceivable that on switching from aerobic to nitrate (anaerobic) growth the synthesis of the components of the oxidase (except cytochrome c_4) is switched off thus thus producing fewer sites for cytochrome c_4 binding. A similar effect may be seen if the model proposed in Figure 9 was in operation although a decrease in the reductase may also be required since cytochrome o was proposed to bind to both the oxidase and reductase simultaneously.

The cytochrome c_4 reductase is unknown but it is conceivable that a cytochrome c_1 may be involved. Hauska (1983) proposed that all species having a third energy conserving site possess a cytochrome c_1. Thus if cytochrome c_4 is involved in transferring electrons to an oxidase it may be that it accepts electons from a cytochrome c_1. It is not known if Pseudomonas stutzeri 224 possesses cytochrome c_1 but a membrane bound cytochrome of approx. 32000 MW can be seen on SDS gels stained for haem. Therefore, on the basis of molecular weight alone this may be a cytochrome c_1. The quantity of this protein can be seen to be markedly reduced in nitrate grown cells (see Figure 30) and may represent the reduction in binding sites for cytochrome c_4. The effect of growth conditions on the levels of the

cytochrome o is unknown. An explanation of the distribution of cytochrome c_4 between membrane and soluble fractions will only be found when the reductase and oxidase of cytochrome c_4 are identified and the effect of growth conditions on them studied.

(3) Comparison of the results for Pseudomonas stutzeri 224 with those of Kodama (1970): It was felt that the results of Kodama should be considered separately since only the membrane bound cytochrome c_4 was discussed in Kodama's study. Kodama (1970) studied the effects of growth conditions in the bacterium Pseudomonas stutzeri (van Niel strain) ie a different strain from the one used in this study and for clarity it shall be referred to as the "Kodama strain".

Table XVIII(B) summarises the results of Kodama on the effects of growth conditions on the levels of membrane bound cytochrome c-552. This cytochrome c-552 is a dihaem cytochrome of 20000 molecular weight and has an α/β ratio of 1.18 (Kodama and Shidara, 1968) indicating that it may well be cytochrome c_4. Kodama's results show induction of cytochrome c-552 when cells are grown anaerobically on nitrate or nitrite. For comparison of results with the results of this study the anaerobic plus nitrite should be considered since in this study the cytochrome c_4 was quantitated at the end of the growth and not at the end of the first phase of anaerobic growth where nitrate is reduced to nitrite, the second phase reducing nitrite with concomitant induction of cytochrome cd_1 (Kodama, 1969).

Kodama's results indicate that cytochrome c-552 is strongly induced when cells are grown anaerobically utilising nitrite as compared to the level of cytochrome c-552 in aerobically grown cells. However, Kodama quantitated cytochrome c-552 from difference spectra of membranes and therefore it cannot be assumed that the absorption peak at 552nm can be attributed to cytochrome c-552 alone. For example, in Pseudomonas stutzeri 224 a membrane c-type cytochrome is induced when cells are grown on nitrate (see Figure 30) which will add to difference spectra in the region of interest. It may be that a similar induction occurs in the "Kodama strain" and it is this induction which produces an apparent induction of cytochrome c-552. Therefore, the quantitation by purification of individual cytochromes should be more reliable since various protein chemical tests can identify that the cytochrome quantitated is pure and the cytochrome of interest.

(4) Quantitation of cytochrome c_4 from haem stained gels and Western blots: Attempts at quantitating cytochrome c_4 from haem stained gels was complicated due to lack of resolution of cytochrome c_4 from other c-type cytochromes, particularly in nitrate membranes (see Figures 30 and 31). Western blotting was attempted in order to solve the resolution problem. However, the antiserum obtained was found to be contaminated with an antibody which reacted with a Pseudomonas stutzeri membrane protein. Unfortunately this membrane protein had a mobility on SDS-PAGE similar to that of cytochrome

c_4 and the two stained bands could not be easily resolved by the gel scanner. For quantitation of the cytochrome c_4 a monoclonal antibody would have to be produced or a better polyclonal antiserum which will react with the cytochrome c_4 alone.

From the haem stained gel (Figure 30) it can be seen that the levels of cytochrome c_4 in the nitrate periplasm is greater than that in the aerobic periplasm and it appears that the level of cytochrome c_4 in aerobic membranes is greater than that in nitrate membranes, a result which is consistent with the purification results.

B. Location

The purification results and the haem stained gel (Figure 30) have demonstrated that cytochrome c_4 is mainly associated with the membrane in Pseudomonas stutzeri 224. Purification experiments have demonstrated that cytochrome c_4 from Azotobacter vinelandii is mainly associated with the membrane while the cytochrome c_4 from Pseudomonas aeruginosa is almost exclusively associated with the membrane (Pettigrew and Brown, 1988). Spheroplasting of Pseudomonas stutzeri 224 and analysis of the resulting fragments revealed that the soluble cytochrome c_4 was associated with the periplasmic fraction.

The experiments mentioned above demonstrated that cytochrome c_4 is mainly associated with the membrane but does not reveal to which side of the membrane it is

attached. It was demonstated, using right-side-out membrane vesicles, that the cytochrome c_4 is found on the periplasmic face of the membrane, as predicted for the general case by Wood (1981). A full discussion of these experiments can be found in Chapter V and therefore will not be repeated here.

It is important to note that the models of cytochrome c_4 function proposed in Chapter I and Section II of this chapter require cytochrome c_4 to be associated with the periplasmic face of the membrane therefore , it was essential to prove that cytochrome c_4 was indeed located on the periplasmic face of the membrane.

CHAPTER VIII : DISCUSSION

Section II - Redox Titration of Cytochrome c_4

A. Models of reduction of cytochrome c_4

The redox titration of cytochrome c_4 produced a sigmoidal Nernst plot which was analysed in terms of two components, ie the two haems have different redox potentials. Previous workers, working mainly with Azotobacter vinelandii cytochrome c_4, had reported only one redox potential. For example, Yang et al (1979) titrated Azotobacter vinelandii cytochrome c_4 and fitted a line of 60mV to the data points and reported a midpoint potential of +260mV. However, closer examination of the published Nernst points reveal that the points do not lie on the line of 60mV slope per unit change in log ox/red but in fact show a sigmoidal shape. Leitch et al (1985) (see Appendix) titrated cytochromes c_4 from Pseudomonas stutzeri 224, Pseudomonas aeruginosa and Azotobacter vinelandii and demonstrated that all the Nernst plots were sigmoidal. The degree of sigmoidicity was dependent on the difference in the redox potentials between the haems. Cytochrome c_4 from Pseudomonas stutzeri shows a difference of approximately 110mV and shows a large sigmoidal effect while cytochrome c_4 from Azotobacter vinelandii, with a difference of approximately 50mV shows a slight sigmoidal shape.

Sigmoidal Nernst plots could arise in two ways: (1) the haems are intrinsically different or (2) the haems

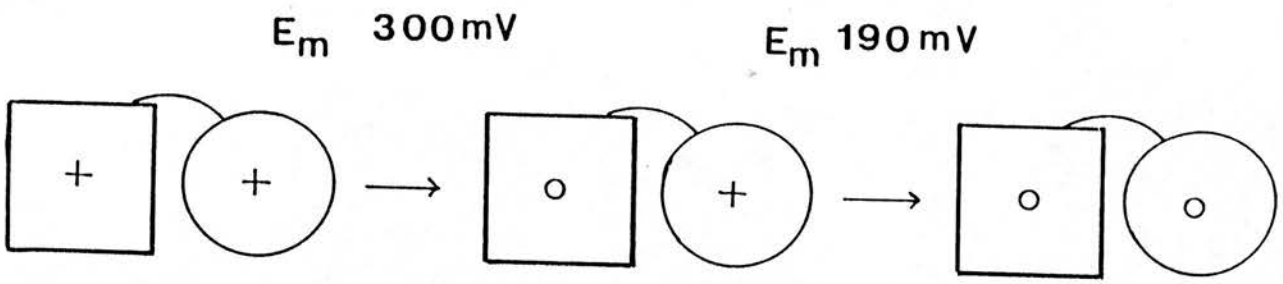
are identical in the oxidised state but reduction of one haem makes reduction of the second haem more difficult. The models are shown in Figure 55 and will be discussed more fully below.

(1) The haems are intrinsically different : Figure 55 (a) shows the model for reduction of cytochrome c_4 assuming the haems are intrinsically different, ie the "square" domain having a midpoint potential of +300mV and the "circular" domain having a midpoint potential of +190mV. Such a model of reduction would fit in well with the proposed flow of electrons from reductase to oxidase without the involvement of a soluble c-type cytochrome (proposed in Figure 9). The domain with the +190mV midpoint potential would interact with the reductase and the +300mV domain with the oxidase. This model would require an intramolecular transfer of electrons from the +190mV haem to the +300mV haem. However, it should be noted that such an intramolecular transfer would not conserve the 110mV of potential energy and would therefore not benefit the bacterium. In addition the proposed x-ray structure of cytochrome c_4 (see Figure 8) suggests that the haems are not orientated in the correct manner for this model to work (L. Sawyer, unpublished). Before totally rejecting the model proposed in Figure 9 solely on the basis of the x-ray work it should be noted that purified (ie not membrane bound) cytochrome c_4 was studied and it may be that the cytochrome c_4 structure is different when attached to

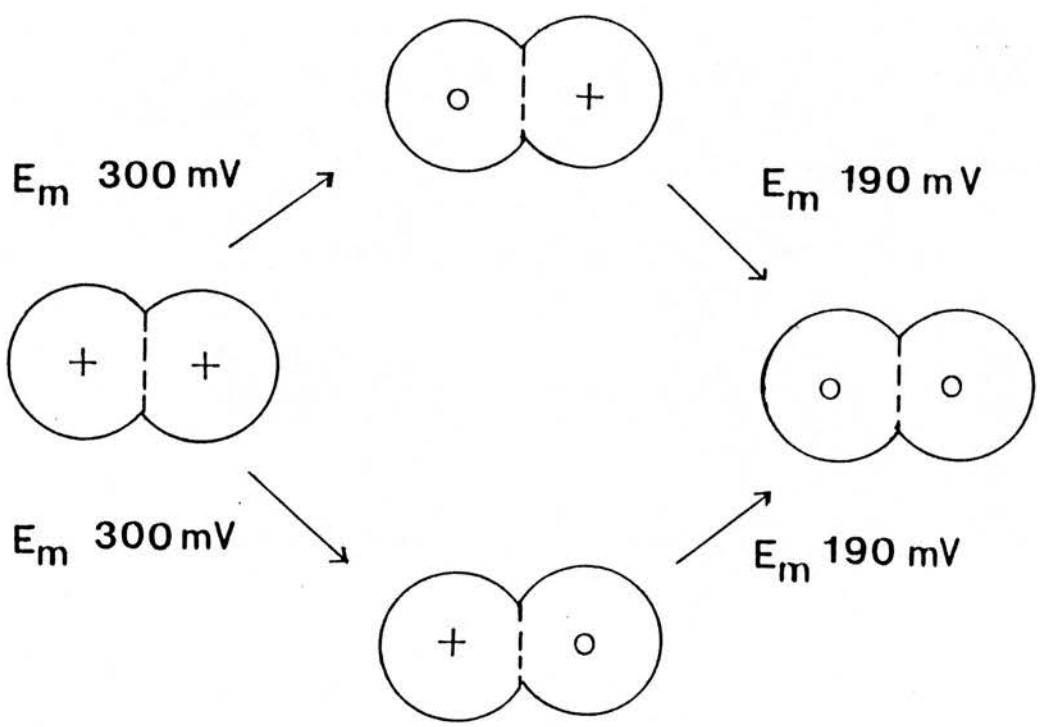
Figure 55 : Possible Models of Reduction of Cytochrome c_4

(a) model where the haems are intrinsically different and (b) where the haems are identical in the oxidised state.

A



B



the membrane (discussed in the Introduction, see Figure 10).

(2) The haems are identical in the oxidised form : Figure 55 (b) shows the proposed scheme for reduction of cytochrome c_4 when the haems are identical in the oxidised form (ie both have a midpoint potential of +300mV). Reduction of one of the haems alters the redox potential of the other (lowers it to +190mV) by some form of interaction between the two domains which may be (a) electrostatic or (b) conformational or (c) a combination of (a) and (b). This lowering of the midpoint potential makes it more difficult to reduce the second haem, ie an example of negative cooperativity.

(a) Electrostatic interaction : In c-type cytochromes the ferrihaem has a formal charge of +1 and the ferrohaem a formal charge of zero thus in fully oxidised cytochrome c_4 both haems will possess a formal charge of +1 (Figure 55 (b)). Therefore there may be charge repulsion between the haems. It is possible to calculate the E_m due to interaction between two positive charges , distance R_{12} apart, across a dielectric (Coulomb's Law). The equation for the Coulomb analysis is shown, and annotated, in Figure 56. Thus for cytochrome c_4 with a distance between the two charges (Fe of the haems) of 19 Å (L. Sawyer, personal communication), a E_m of 38mV was calculated. This value does not account for the whole potential difference (110mV in the case of Pseudomonas stutzeri cytochrome c_4) suggesting an additional factor would be required in the interaction between the haems.

Figure 56 : Coulomb's Equation

$$\frac{N\, Q_1\, Q_2\, e^2}{nF\ 4\pi\ \varepsilon_o\ R_{12}\ Deff}$$

Where N is Avogadro's Number, Q is the charge number, eelectronic charge, F is the Faraday, ε_o is the permittivity of free space, R_{12} is the distance between charges (here 19×10^{-10}m) and Deff is the effective dielectric (here 20).

The dielectric of 20 corresponds to the effective dielectric between the haem propionates and the haem iron calculated for Pseudomonas aeruginosa cytochrome c-551. Experimental calculation of the effective dielectric involves determination of the effect of ionisation of the haem propionic acid 7 on the redox potential of the cytochrome. For cytochrome c-551 this ionisation yielded a fall of 65mV and from Coulomb's law an effective dielectric of 20 was calculated (Moore et al, 1980). The value of 20 was used for cytochrome c_4 since each domain is thought to be structurally similar to a Class I cytochrome and the two domains interact in the region of the haem propionates (Figure 8).

It should be noted that stable dimers of monohaem c-type cytochromes do exist, eg cytochrome c-554(548) from Paracoccus sp. (Hori, 1961) and cytochrome c_5 from Azotobacter vinelandii (Swank and Burris, 1969). The x-ray structure of cytochrome c_5 from Azotobacter vinelandii has been determined and revealed a 16.4Å separation between the haem irons (Carter et al, 1985) which is in fact closer than the haem irons in cytochrome c_4. The redox titration of cytochrome c_5 from Pseudomonas stutzeri shows a Nernst plot to which a straight line of 60mV can be fitted (not shown). In addition the Paracoccus sp. cytochrome c-554(548) has been titrated (Leitch et al, 1985) which also shows a 60mV line. These results imply that no interaction is taking place between the haems of both cytochrome c_5 and cytochrome c-554(548) but they should not be taken to dismiss interaction between haems of cytochrome c_4 but rather to show that haems which are close together need not interact.

(b)Confomational change : It may be that reduction of one of the domains causes a conformational change in that domain which in turn disrupts the interdomain interaction resulting in the lowering of the redox potential of the second haem. It is known that for tetrahaem cytochrome c-554 the four haems interact magnetically (Andersson et al, 1986) and that they all have the same redox potential, +20mV (Miller and Wood, 1983). Dispirito et al (1987) demonstrated that the reduction of cytochrome c-554 was biphasic, the first

phase (fast), accounting for 50% of the total reduction, was followed by a second (slower) phase. This second phase has a relatively high energy of activation suggesting the possibility of a conformational change associated with the reaction. It was also suggested that during cytochrome c-554 function two of the haems are maintained in a particular state of reduction or oxidation, poising the cytochrome at a particular redox potential, while the other two haems function in electron transfer. It is possible that cytochrome c_4 may function as a single electron carrier in which one of the haems is in constant state of reduction.

(3) Negative cooperativity and cytochrome c_4 function : Before discussing what advantages negative cooperativity could confer on cytochrome c_4 it would seem appropriate to briefly define negative cooperativity. To aid discussion of the definition and significance of negative cooperativity the enzyme glyceraldehyde 3-phosphate dehydrogenase (an enzyme of the glycolytic pathway) will be used as an example. Conway and Koshland (1968) have studied the binding of NADH (diphosphopyridine nucleotide) to glyceraldehyde 3-phosphate dehydrogenase and demonstrated the presence of four sites for NADH. The binding of one DPN makes the binding of the second more difficult and so on. It has been proposed that the "loose" sites (eg site 4) possesses greater activity than the tight sites (eg site 1) and it has indeed been shown for glyceraldehyde 3-phosphate dehydrogenase that the K_m and V_{max} suggest

that the specific reactivity is greater for site 4 than for site 2 and that the turnover number for site 4 is twenty times greater than for site 2. This implies that the successive binding of ligand produces, successively, new sites of lower affinity but higher turnover number. Thus during normal conditions, when substrate levels are high, site 4 is utilised in carrying out the reaction whereas sites 1-3 act as reservoirs (NADH constantly bound) to be utilised when substrate levels fall. For example, if the substrate levels fall then a site with a higher affinity for the substrate would be utilised, eg site 3.

Enzymes which exhibit negative cooperativity are found at branch points in enzyme pathways where they are thought to maintain a constant rate of reaction even if the substrate levels fluctuate.

When considering cytochromes the ligand is the electron which does not exist in free state but bound to other redox proteins. During the discussion of cytochrome c function the "substrate" will be reduced cytochrome c reductase and the "product" is the transfer of the electron to the oxidised oxidase. Cytochrome c_4 is the enzyme which possesses two sites of different affinities for electrons, the +300mV domain being the high affinity site and the +190mV domain being the lower affinity site.

It is important, therefore, to discuss the effect of the different midpoint potentials on the rate of electron transfer. Theoretical analysis of electron

transfer reactions by the outer sphere mechanism predicts that there should be a relationship between the rate of electron transfer and the redox potential difference between donor and acceptor. Indeed Meyer et al (1983) and Meyer et al (1984) have shown that the rate of electron transfer between the donors lumiflavin and flavin semiquinones and the acceptor, cytochromes c of different redox potential is dependent on the difference in redox potential, ie the greater the difference between the donor and acceptor the higher the rate.

If such a phenomenon occurs between two redox proteins then it could be imagined that in cytochrome c_4 the rate of reduction of the +300mV domain would be faster than that of the +190mV domain. The converse would of course be true for the oxidation of the haems, ie the rate of oxidation of the +190mV domain would be faster. It may be that the overall rate of electron transfer may be unaffected no matter which haem is utilised in the electron transfer pathway from reductase to oxidase. But, why would such a mechanism be required? It could be imagined that if the bacterium was using a growth substrate which supplied a large quantity of electrons for the electron transfer chain the amount of "substrate" (reduced cytochrome c reductase) would be high allowing maintenance of full reduction of the +300mV haem and utilising the +190mV haem to carry out electron transfer to the oxidase. However, if a different substrate was used which produced a lower

amount of electrons the +300mV haem would be used while the +190mV haem would be constantly in the oxidised state. Therefore it would be the level of reduction of the redox proteins to the reducing side of cytochrome c_4 which would determine which haem is used.

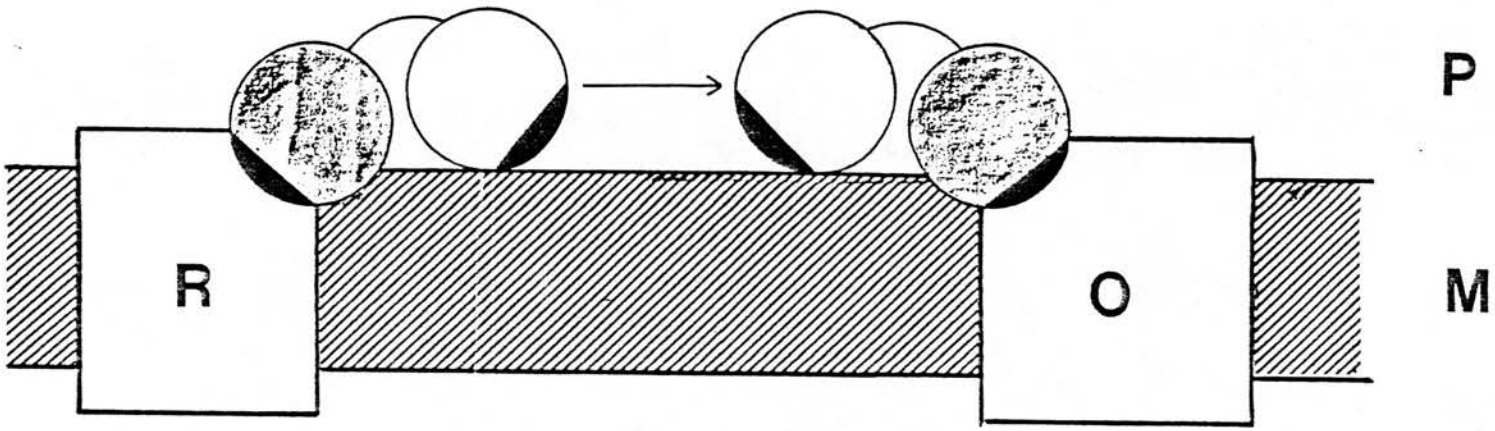
Such a mechanism would work no matter which model of cytochrome c_4 reduction was correct. This model of cytochrome c_4 function requires that the same haem which is reduced by the reductase is also oxidised by the oxidase (see Figure 57) and thus eliminates the energy wasting intramolecular electron transfer required for the model proposed in Figure 9.

It should be pointed out that some authorities state that the potentials of redox proteins must be matched for efficient electron transfer to take place (Moore and Williams, 1976. This may suggest that the two domains of cytochrome c_4 have different acceptors and/or donors, selection of which could depend on the growth conditions. This may also suggest that cytochrome c_4, like glyceraldhyde 3-phosphate dehydrogenase in the glycolytic pathway, is located at a branch point in the electron transfer chain where negative cooperativity is thought to be beneficial.

(4) Models not requiring negative cooperativity: Yang et al (1979) proposed that cytochrome c_4 is an integral component of the terminal oxidase cytochrome o. Since cytochrome c_4 has the potential to deliver a pair of electrons it may be involved (along with the two b-type haems of cytochrome o) in the four electron

Figure 57 : Electron Transfer from Reductase to Oxidase Via Cytochrome c_4

Tentative model of electron transfer where the shaded domain is reduced by the reductase (R) and oxidised by the oxidase (O) with no intramolecular electron transfer step.


R
O
P
M
C

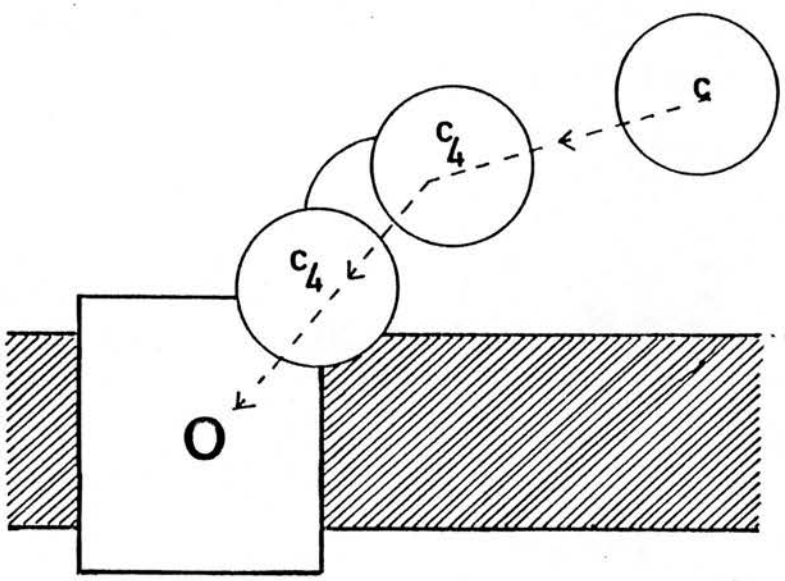
reduction of oxygen. Such a model would require intramolecular electron transfer since electrons would enter the complex through one of the haems.

Another possilble mechanism of cytochrome c_4 function is proposed in Figure 58 where a soluble c-type cytochrome would be required to donate an electron to one of the haems. This mechanism, however, requires an intramolecular transfer of electrons to the other haem for eventual passage to the oxidase.

(5) Experimental strategy for determination of the role of cytochrome c_4 in the terminal region of the electron transfer chain: Yang et al (1979) proposed that cytochrome c_4 is a component of cytochrome o from Azotobacter vinelandii. Jurtshuk et al (1981) purified this oxidase and found it was composed of b- and c-type haems and claimed that the c-type cytochrome c_4 was cytochrome c_4. However, full characterisation of the c-type cytochrome associated with the oxidase needs to be carried out and the properties compared with those of cytochrome c_4. Attempts are being made by Dominic Hunter (PhD student) to purify the o-type oxidase from Pseudomonas stutzeri 224.

Dominic Hunter (personal communication) has successfully removed cytochrome c_4 from the membrane of Psedomonas stutzeri 224 and shown that virtually 100% of the ascorbate TMPD oxidase activity remains (in the absence of soluble c-type cytochromes). Since ascorbate TMPD works via c-type cytochromes this suggests that the c-type cytochrome is still associated with the oxidase.

Figure 58 : Tentative Model of Electron Transfer Utilising both Cytochrome c_4 and a Soluble c-type Cytochrome



P

M

C

If this is the case this c-type cytochrome cannot be cytochrome c_4 since cytochrome c_4 can be shown by SDS-PAGE to have been totally removed. Attempts were made, by studying the duroquinol oxidase, to demonstrate that cytochrome c_4 is involved in transfering electrons to the oxidase without involvement of soluble c-type cytochromes. Duroquinol donates electrons to the reducing end of the electron transfer chain. Removal of cytochrome c_4 from the membrane reduces the duroquinol oxidase activity to 50%. Attempts to reconstitute the duroquinol oxidase by adding back cytochrome c_4 did not increase the duroquinol oxidase above the 50% level although SDS-PAGE shows that cytochrome c_4 is associated with the membrane. It is probable that cytochrome c_4 would be associated with its correct redox partners since during synthesis cytochrome c_4 would be extruded into the periplasm and from there it would then associate with its redox partners. The result with the duroquinol suggests that cytochrome c_4 may act as a "bridge" between reductase and oxidase (as proposed in Figure 9).

Experiments have also been carried out removing cytochrome c_4 from the membranes of Azotobacter vinelandii (Ranald Stuart, personal communication). 100% of the cytochrome c_4 was removed from the membrane but only 50% of the ascorbate TMPD oxidase activity was retained. 100% activity was not recovered when cytochrome c_4 was added back. It was found that 90% of the cytochrome c_4 was added back but only 50% of the

activity remained. It is therefore suggested that some factor other than cytochrome c_4 is involved in reducing the ascorbate TMPD oxidase activity to 50%. Removal of 100% of the cytochrome c_4 with 50% activity remaining tends to suggest, as with Pseudomonas aeruginosa that cytochrome c_4 is not an integral part of the cytochrome o.

B. Experiments to test the models of cytochrome c_4 reduction

An attempt was made to distinguish between the two proposed models of cytochrome c_4 reduction (ie intrinsically different haems or haems having the same redox potential in the oxidised state) by proteolytically cleaving the cytochrome in the connecting loop between the domains, purifying the domains and characterising them individually. However, the results of Chapter IV suggest only one of the domains may be undamaged (by the presence of the 695nm band and a molecular weight of approximately 11000). The observed redox potential of this domain does not correspond to either of the redox potentials of the native cytochrome c_4. This may not be surprising since it is possible that on separation of the domains the structure of the individual domains may be changed thus altering the redox potential. The other domain was found to be damaged (ie the 695nm band was absent and also a 30 amino acid section of the polypeptide chain was missing). The proposal was that the chymotrypsin cleaved

the cytochrome c_4 in the connecting loop, giving rise to an intact fragment and a small fragment with a ragged N-terminus. To enable identification of the correct model of reduction both domains must remain intact so that titration of the two domains would reveal either two different midpoint potentials or that each domain had the same potential.

An attempt was made to titrate an unfractionated mixture of cytochrome c_4 digested with chymotrypsin in the hope that even if the chymotrypsin had nicked the chain to produce the 30 amino acid "missing" chain the cleaved chain may still be associated with the remainder of the domain and thus preserving the structure around the haem. The titration produced the two potentials that were seen in the purification experiment (+115 and -190mV) but in addition a midpoint potential of +225mV was observed. However, the identity of this +225mV potential is uncertain (this is discussed fully in Chapter VI Section III). Thus titration of the mixture only produced the fragments that were seen during the purification experiments ie only one "intact fragment" with a potential corresponding to that of the purified large fragment.

It was therefore impossible to determine whether the haems are identical in the oxidised state or whether they are intrinsically different. An attempt was also made using subtilisin (not shown) to obtain two intact fragments but this protease also produced fragments with potentials of +110mV and -190mV ie the same as the

chymotryptic fragments. Therefore, it appears that some non-destructive method is required to study the redox behaviour of the two haems, eg NMR or MCD on the intact cytochrome c_4.

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APPENDIX

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Complexity in the redox titration of the dihaem cytochrome c_4

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Key words: Cytochrome c_4; Redox titration; (*P. aeruginosa*, *P. stutzeri*, *A. vinelandii*)

Redox titration of the dihaem, two domain cytochromes c_4 from *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Azotobacter vinelandii* showed complex behaviour indicative of the presence of two redox components. In the case of the *P. stutzeri* cytochrome c_4, two spectroscopically distinct components were present during the redox titration. In contrast, cytochrome *c*-554(548) from a halophilic *Paracoccus* species is a stable dimer of a monohaem cytochrome which shows close homology to cytochrome c_4, but does not show complexity in its redox titration. The presence of chemically distinct haem environments or anti-cooperative interactions between identical haem groups are two possible explanations for the redox complexity of cytochrome c_4. The simple redox titration of cytochrome *c*-554(548) shows that haems situated relatively close together need not interact, but direct cleavage, separation and study of the domains will be necessary to decide whether they do or do not interact in the case of cytochrome c_4.

Introduction

Cytochrome c_4 is a dihaem cytochrome of an approx. molecular weight of 20000. The amino acid sequence shows evidence of a gene duplication event [1,2] giving rise to a two domain protein [3], each domain resembling a typical small monohaem cytochrome *c*.

Cytochrome c_4 is poorly characterised with respect to distribution, cellular location and electron-transfer function. This may be due in part to its probable location in the membrane and also to the fact that its characteristic properties have not always been clearly recognised; cytochrome c_4 is distinguished by a molecular weight of 20000 and a low α/β absorption ratio. On the basis of these properties, members of the cytochrome c_4 group have been isolated from several denitrifying bacteria [4–7]. The suggestive correlation with the denitrification process, however, is complicated by the presence of cytochrome c_4 in the strict aerobe *Azotobacter vinelandii* where it is proposed to form a terminal cytochrome oxidase complex with cytochrome *o* [10,11]. Such a complex may be widespread [12–15] and the apparent correlation between cytochrome c_4 and denitrification may simply be a consequence of the induction of cytochrome *o* under conditions of limiting oxygen.

The dihaem nature of cytochrome c_4 raises interesting questions regarding its role in electron transfer. Terminal oxygen reductases seem to be designed in such a way as to avoid single electron-reduction steps [16], and cytochrome c_4 may be a device for ensuring coordinated delivery of two electrons. A second possibility is that one domain of cytochrome c_4 may bind to a membrane reductase, while the other domain binds to an oxidase. In such a mode, cytochrome c_4 would

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Abbreviations: PMS, phenazine methosulphate; DAD, diaminodurol; Cyt, cytochrome.

provide a conduction route via intramolecular electron transfer.

Redox complexity in a dihaem system may be due to intrinsic redox potential differences or to haem : haem interactions giving rise to deviations from simple Nernstian behaviour. Such deviations are present in cytochrome c_4 titrations in the literature (see for example Fig. 1(a) of Ref. 10), but have not been noted and commented on. This paper describes the redox complexity of three cytochromes c_4 from *Pseudomonas aeruginosa, Pseudomonas stutzeri* and *A. vinelandii.*

Methods

Organisms and cytochromes

The organisms used were *P. aeruginosa* (P6009), *P. stutzeri* (Stanier 224, ATCC 17591) and *A. vinelandii* (strain *o*, ATCC 12837). Cytochrome c_4 was extracted from membranes using butanol [8] and purified by methods to be described separately (unpublished data). All three purified cytochromes c_4 gave single bands after SDS polyacrylamide gel electrophoresis and in the cases of the *P. aeruginosa* and *A. vinelandii* cytochromes the amino acid compositions closely resembled those of the published amino acid sequences [1,2]. The amino acid sequence of the cytochrome from *P. stutzeri* has not yet been determined.

Cytochrome *c*-554(548) from a halophilic *Paracoccus* species (originally described as *Halotolerant micrococcus* [17]) was a kind gift from Dr. T.E. Meyer. Horse cytochrome *c* was from Sigma (Type VI).

Redox titrations

Reductive and oxidative titrations were performed in an anaerobic cuvette constantly bubbled with argon and magnetically stirred (Cell stirrer, Bel-Art). The cuvette contained approx. 5 μM cytochrome in 0.02 M sodium phosphate (pH 7), 20 μM phenazine methosulphate (PMS, Sigma), Diaminodurol (DAD, Aldrich) and ferric ammonium sulphate and 0.4 mM EDTA.

The ambient redox potential (E_{obs}) was monitored by a Pt pin electrode in combination with an Ag|AgCl reference (Russell pH Ltd., Auchtermuchty, U.K.) and the potential with reference to the standard hydrogen electrode (E_h) was obtained by adding 198 mV to E_{obs} [18].

Oxidative and reductive titrations were performed by addition of 0.02 M potassium ferricyanide and 0.02 M sodium dithionite, respectively. The ambient potential reading stabilised within a minute of an addition and the spectrum was then recorded. Full reduction was achieved by addition of solid dithionite. Isosbestic points were maintained throughout the titration except after addition of solid dithionite, where the reduction of PMS gives rise to a small contribution to the cytochrome spectrum. This spectrum was therefore offset to match the isosbestic point established during the titration.

Redox titrations were also performed by the method of mixtures in known concentrations of potassium ferro- and ferricyanide without a redox electrode present. Cytochromes were reduced with a 100 × excess of sodium ascorbate, and the reducing agent was removed by passage down Sephadex G-25 equilibrated in 0.5 mM sodium phosphate (pH 7) containing 10 mM NaCl and 0.5 mM EDTA. Oxidative, aerobic titrations were carried out by additions of small portions of 2.5 mM potassium ferricyanide to a solution containing 8 μM cytochrome in 1.7 mM sodium phosphate/1 mM EDTA/0.5 mM potassium ferrocyanide. The ambient redox potential was calculated for each addition after accounting for ferricyanide reduced during oxidation of the cytochrome. The ionic strength was approx. 0.02 M and did not alter significantly during the ferricyanide titration permitting the use of an E_m value of 385 mV for the ferro-ferricyanide couple given in Ref. 19 for this ionic strength.

Results

Horse cytochrome c and cytochrome c-558(548) from halophilic Paracoccus sp.

In both cases, isosbestic points and the position and shape of spectral peaks did not change throughout the redox titration (Fig. 1a shows the titration of the cytochrome *c*-554(548)). The experimental Nernst data fitted a slope of 60 mV (Fig. 2) with midpoint potentials at pH 7.0 of 261 mV for horse cytochrome *c* and 195 mV for cytochrome *c*-554(548) (Table I). In some samples of horse cytochrome *c*, downward deviations from

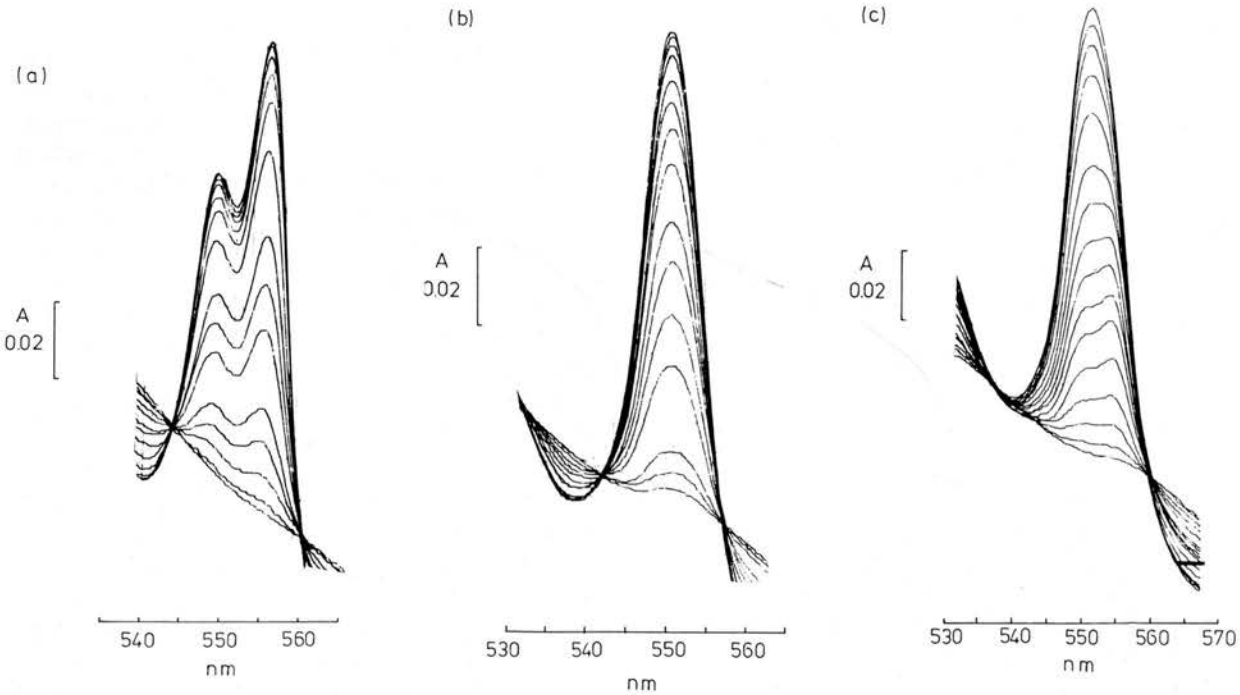

Fig. 1. Cytochrome spectra were recorded on a Unicam SP1800 spectrophotometer adapted to take a stirred anaerobic cuvette with a redox electrode. The cuvette contained approx. 5 μM cytochrome in 0.02 M sodium phosphate (pH 7), 20 μM PMS, DAD and ferric ammonium sulphate, and 0.4 mM EDTA. The redox titration was performed at 23°C. Each spectrum corresponds to a particular ambient redox potential (not shown) and is used to calculate log $Cyt._{ox}/Cyt._{red}$. Titration by the method of mixtures gave very similar results (experimental points not included). (a) Cytochrome *c*-554(548) from halophilic *Paracoccus* sp.; (b) cytochrome c_4 from *A. vinelandii*; (c) Cytochrome c_4 from *P. stutzeri* 224.

TABLE I

ANALYSIS OF THE NERNST PLOTS OF CYTOCHROMES

The analyses are for the theoretical curves shown in Figs. 2 and 3. The percentage contribution in the case of *P. stutzeri* cytochrome c_4 was calculated for the absorbance change at 552 nm, but the figures are little different if the absorbance changes at the α-peak maxima are used.

Cytochrome		Midpoint potentials (mV)	Percentage contribution
Horse cyt. *c*		261 (single)	100
Cyt. *c*-554(548)		195 (single)	100
Cyt. c_4	*A. vinelandii*	317	56
		263	44
	P. aeruginosa	322	50
		268	50
	P. stutzeri	300	41
		190	59

the 60 mV line were observed at logarithmic oxidation/reduction values more negative than −1 indicating the presence of small amounts of cytochrome with a more negative midpoint potential. This problem could be overcome by passage down Sephadex G-75 superfine (1.5 × 90 cm in 0.02 M Tris-HCl (pH 8.0) containing 0.1 M NaCl).

Cytochrome c_4 from A. vinelandii and P. aeruginosa

Isosbestic points and spectral characteristics did not alter during the titrations (Fig. 1b shows the titration of *A. vinelandii* cytochrome c_4), but in both cases the experimental data clearly lay off the 60 mV line expected for a single redox component (Fig. 3a and b).

The simplest model to account for this deviation is that the two haems differ in redox potential. Theoretical curves can be drawn for the case where two different components contribute to a

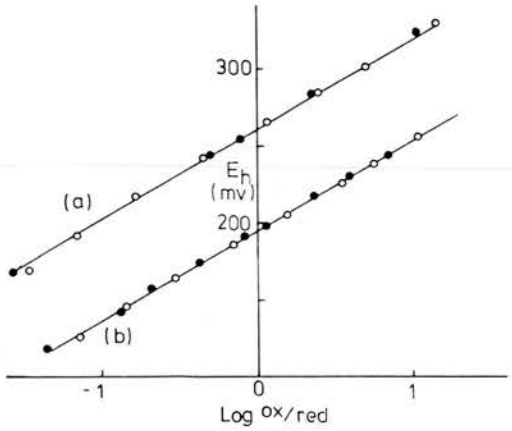

Fig. 2. Nernst plots for horse cytochrome *c* and cytochrome *c*-554(548) from halophilic *Paracoccus* sp. ○, ●, Experimental points from oxidative and reductive titrations respectively. Values of log $Cyt._{ox}/Cyt._{red}$ were obtained from the absorbance changes at the α-peak maximum. The lines are slopes of 60 mV. (a) Horse cytochrome *c*; (b) cytochrome *c*-554(548) from halophilic *Paracoccus* sp.

redox titration; in constructing such a theoretical curve, the relative contributions of the two components to the absorption spectra are estimated from the anti-logarithm of the logarithmic values on the total oxidation/total reduction scale at the middle of the sigmoidal portion of the data. A simple programme written in BASIC for a BBC microcomputer, and available on requests from the authors, allowed rapid visual assessment of trial fits to the experimental data. The solid curves in Fig. 3a and b are theoretical lines, defined by the parameters in Table I, which provide a good visual fit to the data. In all three titrations in Fig. 3, the experimental points at greater than 90% reduction fall slightly off the theoretical curves for two components. This can be corrected by proposing the presence of a small amount of cytochrome (approx. 5%) of lower redox potential. Since the samples are not contaminated by other cytochromes as judged by electrophoresis, this probably represents a small proportion of cytochrome c_4 damaged perhaps as a consequence of butanol extraction. Indeed cytochrome c_4 prepared in low yield from the soluble fraction of cells disrupted by the French press does not show this deviation from a two-component curve at high levels of reduction.

Cytochrome c_4 from P. stutzeri

In contrast to the other spectroscopic titrations, that of cytochrome c_4 from *P. stutzeri* clearly shows the presence of two spectrally distinct components having different isosbestic points (Fig. 1c). The contribution of the higher potential component with the asymmetric, red-shifted α-peak to the fully reduced spectrum is detectable as an asymmetry on the long-wavelength side of the

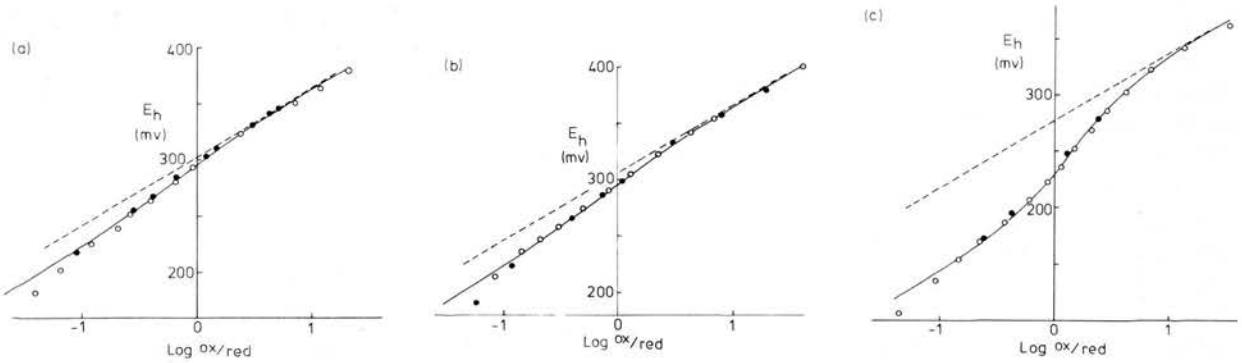

Fig. 3. Nernst plots for cytochrome c_4. ○, ●, Experimental points from oxidative and reductive titrations respectively. Values of log ox/red were obtained from the absorbance changes at the α-peak maximum for the cytochromes c_4 from *P. aeruginosa* and *A. vinelandii*. In the case of *P. stutzeri* cytochrome c_4, log ox/red was calculated from the absorbance change at 552 nm, equidistant between the α-peak maxima of the two components. However, there was very little difference in the analysis if log ox/red was calculated from the absorbance at the α-peak maximum at each stage of the titration. The broken lines are slopes of 60 mV. The solid lines are theoretical curves for two components with midpoint potentials and percentage contributions as defined in Table I. Cytochrome c_4 from (A) *A. vinelandii*; (b) *P. aeruginosa*; (c) *P. stutzeri*.

α-peak. Of the systems studied in this paper (Fig. 3c, Table I) the two haems of *P. stutzeri* cytochrome c_4 show the greatest separation in midpoint potentials.

The Nernst data were calculated at 552 nm, which lies between the α-peak maxima of the two components. However, there was very little difference in the analysis if the absorbance at the α-peak maximum at each stage in the titration was used to calculate the logarithm of the total oxidation/total reduction value.

Discussion

Redox titration of the dihaem cytochrome c_4 indicates the presence of potentiometrically distinct haem groups with a separation varying between 54 and 110 mV. Such complexity could arise in two ways. Either the redox potentials of the haems are intrinsically different (Fig. 4a), or there is interaction between two identical haems such that the addition of the first electron is more favourable (more positive redox potential) than the addition of the second (Fig. 4b). In this respect we should note that the Nernst plot is the formal equivalent of the Hill plot used to analyse cooperativity in ligand binding [20]. Thus Nernst slopes less than 60 mV indicate positive cooperativity, while those greater than 60 mV (as seen here) show negative cooperativity. Such anti-cooperativity might be explained in electrostatic terms as due to charge repulsion of the ferric haems in the fully oxidised protein.

We propose two ways of distinguishing these possibilities. One is to study a related dihaem system with haem environments known to be chemically identical, and to ask if there is evidence of haem interaction. The cytochrome *c*-554(548) of halophilic *Paracoccus* sp. contains 83 amino acids and a single haem group [1]. The amino acid sequence is closely related to the first half of the cytochrome c_4 molecule [1] and the cytochrome is isolated as a very stable dimer [17]. We propose that this dimer of cytochrome *c*-554(548) may structurally resemble the two domains of cytochrome c_4 and that its potentiometric titration may be a test for anti-cooperative effects between haem groups. As can be seen in Fig. 2 no such effects are detected indicating that the haems of

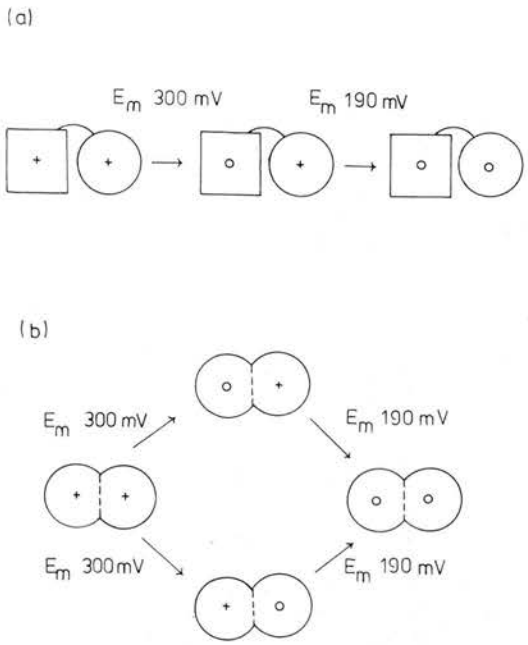

Fig. 4. Models for reduction of cytochrome c_4. In model (a) two domains with intrinsically different redox properties are shown as a square and a circle. The midpoint potential for reduction of the ferric haem (+) in the 'square' domain is 300 mV and for the ferric haem in the 'circular' domain is 190 mV. The domains do not interact. In model (b) the two domains are equivalent in the fully oxidised state. The addition of the first electron can occur to either domain, and alleviates an electrostatic repulsion. Thus the first electron reduction is a more favourable process (more positive redox potential) than the second.

the cytochrome *c*-554(548) dimer do not interact. A similar result was obtained (not shown) for the dimeric cytochrome c_5 of *Pseudomonas stutzeri*.

Although such evidence indicates that two haem groups, separated by the relatively small distance that will prevail in these dimers, need not interact, it does not demonstrate that they do not interact in cytochrome c_4. A more direct approach is to proteolytically cleave the two-domain cytochrome c_4 molecule and study the separated domains. Experiments of this kind are in progress.

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Haem staining in gels, a useful tool in the study of bacterial *c*-type cytochromes

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Key words: Heme staining; Cytochrome-*c* type; Respiration; (Bacterium)

c-Type cytochromes are the only cytochromes to retain quantitatively their haem during SDS gel electrophoresis and can be identified in complex mixtures by their haem peroxidase activity. Although weak staining bands may be due to residual haem attachment to *b*-type cytochromes or to migration of haem, these effects could be abolished by prior extraction with organic solvent. The colour yield of haem staining allowed an estimate of the relative amounts of a particular cytochrome, particularly if loadings were below 50 pmol. At greater loadings, a plateau of colour development was observed. Freshly made gels gave much poorer colour development. The haem-staining method was shown to be useful in three particular areas of study in bacterial respiration. Firstly, it allows assessment of the results of sphaeroplast formation in gram negative bacteria. Secondly, quantitation of the haem stain was useful in the investigation of the induction effects of growth conditions on *c*-type cytochromes. Thirdly, the interpretation of complex chromatographic profiles was greatly simplified by the use of haem-stained SDS electrophoretic gels.

Introduction

Proteins retaining haem in SDS can be detected by their haem peroxidase activity after electrophoresis using 3,3′,5,5′-tetramethylbenzidine (TMBZ) as an oxidisable substrate which gives rise to a blue insoluble precipitate [1]. The method was developed, and has been widely used, for proteins such as cytochrome *P*-450 and cytochromes *b* which contain protohaem IX [1–3], but because this haem is not covalently bound, only a residue is retained under denaturing conditions and this varies depending on the precise conditions employed.

On the other hand, the *c*-type cytochromes contain covalently bound haem and are ideally suited to the application of the peroxidase activity method after SDS electrophoresis, yet it has been relatively little used in this context (a notable exception is the paper of Ward et al. [4]).

In our laboratory the haem-staining method has become a routine and invaluable method in three particular areas in our studies of bacterial respiration. These are the assessment of sphaeroplast formation, the investigation of induction of *c*-type cytochromes and the identification of different *c*-type cytochromes in complex chromatographic profiles. In view of a recent paper [5] pointing out a potential shortcoming of the method, we thought it appropriate to present a paper emphasising the considerable merits of the method and commenting on the ways in which problems of interpretation can be solved.

Abbreviation: TMBZ, 3,3′,5,5′-tetramethylbenzidine.

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Materials and Methods

Growth of cells and preparation of cell extracts

Pseudomonas stutzeri 224. Aerobic growth of

Pseudomonas stutzeri 224 (ATCC No. 17591) was done in a medium (pH 7) containing 17 mM tri-sodium citrate/7 mM KH_2PO_4/2 mM $MgSO_4 \cdot 7H_2O$/4 g yeast extract (Oxoid) per l. 1-l cultures were incubated for 16 h, at 30°C on an orbital shaker. For denitrifying growth, $NaNO_3$ was added to a final concentration of 58 mM after 5 h of aerobic conditions, the flasks sealed with two layers of Nescofilm and further incubated at 30°C for 16 h. Cells were harvested by centrifugation at 4000 × *g* for 30 min at 4°C (Beckman J-6B fitted with J.S 5.2 rotor) and washed once in 10 mM sodium phosphate (pH 7) buffer and re-centrifuged as above.

Periplasmic, cytoplasmic and membrane fractions were prepared by the method of Wood [6], except that cells were suspended to a final concentration of 100 mg/ml.

Acid-acetone and ethanol-acetone extracts. 10 vols. of 0.01 M HCl in acetone was added to the sample and after 30 min the precipitate was sedimented in a Beckman microfuge. The pellet was then dissolved in 62.5 mM Tris-HCl/2 mM EDTA/2% SDS/10% glycerol (pH 7), ready for electrophoresis.

Ethanol-acetone extraction was carried out similarly using ethanol/acetone (1 : 1).

SDS-polyacrylamide electrophoresis. Electrophoresis was carried out using the buffer system of Laemmli [7] with the addition of 2 mM EDTA.

Gels were cast (% acrylamide and % bis-acrylamide detailed in the figure legends) as slabs (130 × 150 × 1 mm) with a 4% acrylamide stacking gel, and run at 50 V for approx. 16 h, or at 150 V for 4–5h.

Gels were stained for haem by the method of Thomas et al. [1] with the following modifications. Gels were immersed for 30 min in 1.25 mM TMBZ in methanol/0.25 M, sodium acetate (pH 5) (30 : 70) with constant shaking, after which the H_2O_2 was added to 26 mM. After shaking for a further 15 min the staining solution was replaced twice with fresh propanol/0.25 M sodium acetate (pH 5) (30 : 70). Photography and scanning of gels was carried out within 2 h of staining.

The centre of each channel was scanned using a Shimadzu CS-930 gel scanner at 690 nm in transmission mode with a beam of dimensions 0.05 × 2 mm in 0.2 mm steps. Photography of gels was through a yellow filter (Cokin A.001).

Gels were stained for protein with 2.0 g/l Coomassie brilliant blue R; C.1.42660 in methanol/acetic acid/water (4.5 : 1 : 4.5), destained in methanol/acetic acid/water (3 : 1 : 6) and photographed as above.

Ion-exchange chromatography. Samples were desalted into 5 mM Tris-HCl (pH 8) on Sephadex G-25 coarse and adsorbed onto DEAE cellulose (Whatman DE52, 10 × 1 cm) equilibrated with 5 mM Tris-HCl (pH 8). The column was developed with a 5 mM Tris-HCl to 5 mM Tris-HCl + 200 mM NaCl linear gradient (400 ml total volume).

Enzyme assays. Isocitrate dehydrogenase assays were carried out using the method of Bernt and Bergmeyer [8].

Results and Discussion

Assessment of the method

Specificity

The haem of *c*-type cytochromes is covalently bound and quantitatively retained on the protein in SDS. In contrast, most of the haem in proteins containing protohaem IX is dissociated under denaturing conditions and is observed as a rapidly migrating band exhibiting TMBZ peroxidase activity on SDS electrophoretic gels. However, a small and variable portion of protohaem IX is retained by such proteins. We have observed this with myoglobin, *Escherichia coli* cytochrome *b*-562 and yeast cytochrome *c* peroxidase (results not shown) and others have found the same for membrane-bound *b*-type cytochromes [2,3].

In addition to this residual retention of protohaem IX, there may be artefactual transfer to non-haem proteins. For example, ovalbumin will stain for TMBZ peroxidase activity if subjected to gel electrophoresis together with myoglobin (results not shown).

Both the residual retention of protohaem by myoglobin and its transfer to ovalbumin can be minimised or eliminated by prior treatment with organic solvents such as acidified acetone or ethanol/acetone (results not shown). The transfer of haem to ovalbumin can be blocked by treatment with *N*-ethylmaleimide implicating protein sulfhydryl groups.

In a complex sample known to contain both *b*- and *c*-type cytochromes such as the bacterial membranes of Fig. 1, a pattern of haem staining will be obtained which cannot be interpreted without further information. The bands labelled a, b and c are not affected by treatment with *N*-ethylmaleimide or extraction with ethanol-acetone and can therefore be identified as *c*-type cytochromes. The bands labelled 1, 2 and 3 diminish or disappear with *N*-ethylmaleimide treatment or extraction with ethanol-acetone. These bands may represent *b*-type cytochromes with residual haem attachment, but the possibility of artefactual migration of protohaem IX cannot be excluded.

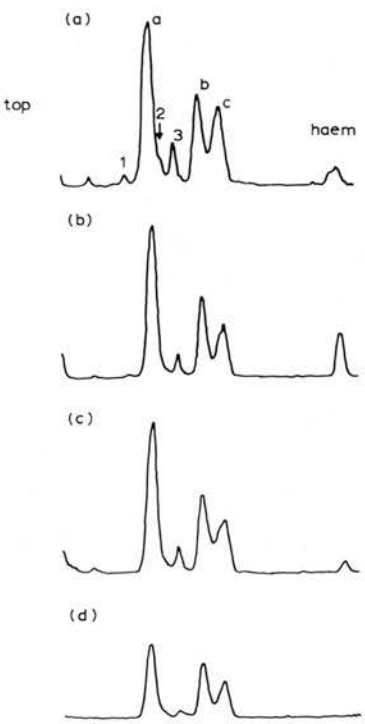

Fig. 1. Haem staining of membrane cytochromes from *Pseudomonas stutzeri*. Electrophoresis was performed in a gel containing 12.5% acrylamide and 0.3% bisacrylamide using spheroplast membrane suspensions of aerobically grown *Ps. stutzeri* 224. Equal amounts of membranes were loaded in each channel, except for (d) where the precipitate after acid-acetone treatment did not fully redissolve in SDS solution. (a) Untreated membranes; (b) membranes + 100 nmol *N*-ethylmaleimide; (c) membranes extracted with ethanol acetone; (d) membranes extracted with acid-acetone. Top indicates loading position of the vertical slab gel.

Extraction with acid-acetone also removes bands 1, 2 and 3 (Fig. 1d), but the protein precipitate dissolves poorly and overall decrease of all bands is observed.

With photosynthetic systems, a further complication is photooxidation of TMBZ by chlorophylls [3]. However, this can be avoided either by prior extraction with ethanol/acetone or by staining in the dark (results not shown).

Miller and Nicholas [5] found that a Cu-containing oxidase showed TMBZ oxidase activity under denaturing gel electrophoresis. Such an activity can easily be distinguished from haem peroxidase activity by examination of the gel before addition of H_2O_2.

With these precautions and controls, electrophoretic bands with TMBZ peroxidase activity can be reliably identified as *c*-type cytochromes. Such an identification can be further confirmed by separation of the individual cytochromes and spectrophotometric characterisation.

Quantitation

Haem staining is a progressive, catalysed reaction rather than a stoichiometric process and this leads to problems of quantitation in a gel. However, the following experiments suggest that, with some precautions, the method can yield useful information on the relative amounts of *c*-type cytochrome present.

The data of Fig. 2 (solid lines) are for a single gel containing different amounts of two *c*-type cytochromes. Colour yield was measured after the standard staining procedure as described in the Materials and Methods. Several comments can be made. First, the two different cytochromes give similar colour yields. Second, the staining is sensitive, detecting 10 pmol of haem *c* with ease. Third, with amounts of haem greater than 50 pmol, a plateau of staining is observed. This may be due either to local exhaustion of TMBZ or H_2O_2 in the gel, although the effect was still observed with doubled concentrations (results not shown). Alternatively, this effect may be due to haem destruction by H_2O_2. Fourth, the colour yield for 100 pmol or less, is much poorer in a freshly prepared gel (Fig. 2, broken lines), perhaps due to residual persulphate or derived peroxides either interfering directly with the haem-staining reaction or pro-

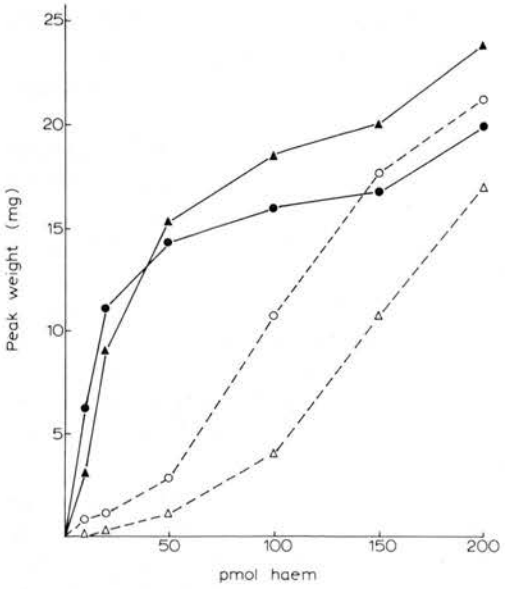

Fig. 2. The variation of colour yield with quantity of purified cytochromes in stored and fresh gels. Electrophoresis was performed in a gel containing 15% acrylamide and 0.4% bisacrylamide on samples containing various amounts of horse cytochrome *c* (●, ○) and *Pseudomonas aeruginosa* cytochrome *c*-551 (▲, △). In one experiment (closed symbols) a gel was used which had been allowed to 'age' for 18 hours: in a second experiment (open symbols) a freshly made gel was used. The central portion of each channel was scanned at 690 nm and the peaks cut from the chart paper and weighed.

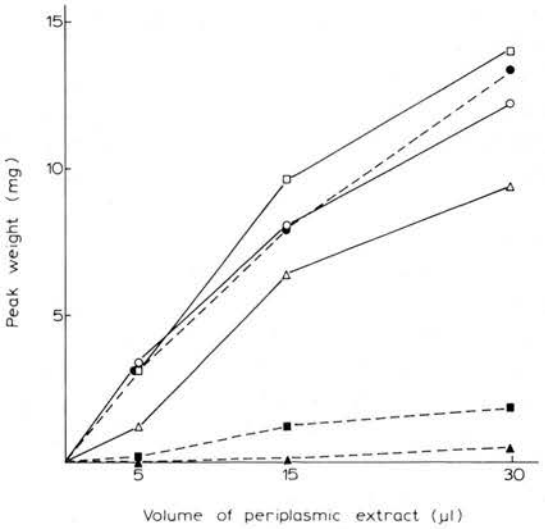

Fig. 3. The variation of colour yield with the quantity of periplasmic extract from *Pseudomonas stutzeri*. Electrophoresis was performed in a gradient gel of 10% acrylamide, 0.2% bisacrylamide to 25% acrylamide, 0.5% bisacrylamide on samples of periplasmic extracts of *Ps. stutzeri*. Individual cytochromes were identified with reference to purified markers and the colour yield associated with each band was expressed as a peak weight as described in Fig. 2. Cytochromes present in aerobically grown cells are denoted by closed symbols: those present in denitrifying cells by open symbols. Cytochrome cd_1 (nitrite reductase) (△, ▲); 30 K cytochrome *c* (□, ■); cytochrome *c*-551 (○, ●).

gressively damaging the haem during electrophoresis.

As we will show in the following section one of the most powerful applications of the haem-staining method is the assessment of induction of bacterial *c*-type cytochromes under different growth conditions. Because of the plateau of haem staining, however, different amounts of a *c*-type cytochrome might give similar colour yields of staining. This problem can readily be overcome by ensuring that loadings lead to colour yields below the plateau region. Fig. 3 shows different loadings of periplasmic samples from aerobic and denitrifying *Pseudomonas stutzeri*. The colour yields for all the bands fall below the plateau region of Fig. 2 (the ordinates are comparable) and the differences in the levels of the cytochrome cd_1 and 30 K cytochrome *c* with the growth conditions are clearly seen at all loadings.

Some workers specifically avoid [1,3] or include [2] reducing agents when running electrophoresis gels for haem staining. In our experience the presence of dithiothreitol leads to poor or negligible haem staining. This is probably due to dissociation of Fe II from the haem to yield a porphyrin and indeed this forms the basis for the fluorescence method of cytochrome *c* detection in electrophoretic gels [9,10].

Applications

Topography of bioenergetic membranes – sphaeroplast formation

The topography of redox centres on either side of, and within, a bioenergetic membrane is an important ingredient of the chemiosmotic explanation of energy conservation. In bacteria, Wood [11] has proposed that *c*-type cytochromes are located either in the periplasmic space or bound to the periplasmic side of the cell mem-

brane. Haem-stained gels of sphaeroplast fractions afford a good means of testing this proposal.

The electrophoretic analysis of the periplasmic, cytoplasmic and membrane-bound compartments of aerobic and denitrifying *Pseudomonas stutzeri* is shown in Fig. 4 and 5. It is clear from Fig. 4, channels 1a and b and 2a and b that the soluble cytochromes *c*-551, cd_1 and 30 K are located in the periplasmic space. The small percentage (6%) of cytochrome *c*-551 found in the cytoplasmic fraction may simply be due to entrapment in the sphaeroplast pellet or to partial spheroplast formation with incomplete release of periplasmic contents until osmotically shocked. The latter is the main problem associated with spheroplast studies in bacteria. In our hands, for example, *Pseudomonas aeruginosa* gives incomplete sphaeroplast formation and the 'cytoplasmic' fraction can contain ass much as 80% of the cytochrome *c*-551. The problem is not always realised and has given rise to confusing proposals for the

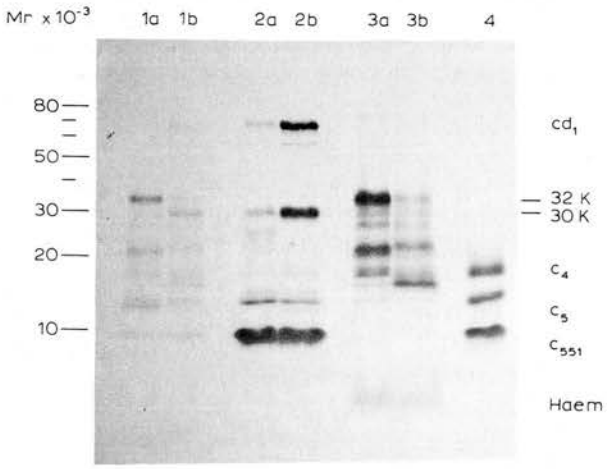

Fig. 4. Assessment of spheroplast formation: haem staining after SDS electrophoresis of cytoplasmic, periplasmic and membrane fractions of aerobic and denitrifying *Pseudomonas stutzeri*. The cytoplasmic (1a, 1b), periplasmic (2a, 2b) and membrane (3a, 3b) fractions were obtained from aerobic (1a, 2a, 3a) and denitrifying (1b, 2b, 3b) *Ps. stutzeri* as described in the Materials and Methods section. These fractions were adjusted to the same volume and equal portions were subjected to SDS electrophoresis as described in Fig. 3. Lane 4 contains 0.1 nmol of purified c_4, c_5 and *c*-551 from *Ps. stutzeri*. The scale of M_r was constructed from the relative mobilities of a set of molecular-weight marker proteins (not shown).

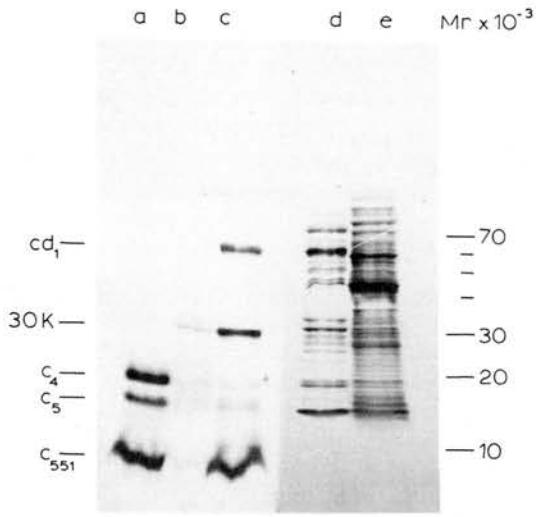

Fig. 5. Assessment of sphaeroplast formation: protein staining after SDS electrophoresis on a 5% acrylamide, 0.1% bisacrylamide to 20% acrylamide, 0.5% bisacrylamide gradient gel of periplasmic and cytoplasmic fractions of denitrifying *Pseudomonas stutzeri*. Lane (a), 0.1 nmol of purified cytochromes c_4, c_5 and *c*-551; lanes (b) and (e): cytoplasmic fraction; lanes (c) and (d): periplasmic fraction. Lanes (a), (b) and (c) were stained for haem. Lanes (d) and (e) were stained for protein with the Coomassie blue reagent. The scale of M_r was constructed from the relative mobilities of a set of molecular-weight marker proteins (not shown).

dual location of, for example, cytochrome c_3 in *Desulfovibrio* [12].

The integrity of the cytoplasmic compartment after the first phase of the sphaeroplast formation cannot be assessed by haem-stained gels and requires the assay of a cytoplasmic enzyme marker. In the present studies only 2–4% of isocitrate dehydrogenase was released into the periplasmic fraction, indicating stability of the spheroplasts formed. The absence of cytoplasmic contamination of the periplasmic fraction and the relatively simple composition of the latter is shown by protein staining after electrophoresis (Fig. 5). There are only a few periplasmic proteins and the 30 K cytochrome and cytochrome cd_1 can be identified as dominant components. The small cytochrome *c*-551 probably leaches out during the prolonged staining and destaining procedure.

The membranes have their own distinctive complement of *c*-type cytochromes (Fig. 4) and the fast-migrating free haem indicates the prob-

able presence of *b*-type cytochromes also. There is perhaps more severe contamination (up to 15%) of the cytoplasmic fraction with membrane cytochromes than with soluble cytochromes and this is probably due to incomplete membrane sedimentation from the viscous DNA-containing sphaeroplast lysate.

Induction of c-type cytochromes

Bacteria are often bioenergetically versatile and can induce appropriate electron-transfer components in response to a changed environment. Most studies on the effects of growth conditions on cytochrome *c* (see, for example, Refs. 13 and 14) simply measure the change in total cytochrome *c* content. However, bacteria often contain several *c*-type cytochromes and individual trends will be lost in the total change. One solution is to purify individual cytochromes *c* (see for example Refs. 15 and 16), but this is time-consuming and differential losses during purification may give misleading results.

Haem-stained gels of soluble and membrane extracts allow examination of the individual cytochromes present in the whole system and, as indicated in section A, an estimate can be made of their relative amounts under different growth conditions. The periplasm of denitrifying *Ps. stutzeri* contains greatly increased amounts of cytochrome cd_1 (a nitrite reductase) and a 30 K cytochrome *c* (Fig. 3 and 4). In the membrane fraction a 32 K cytochrome *c* (which may correspond to the cytochrome c_1-like protein identified in *Ps. aeruginosa*) [17] is the dominant component of aerobic membranes but is almost absent from denitrifying membranes (Fig. 4).

Identification of cytochromes c during purification

As noted above, a bacterial extract will usually contain several *c*-type cytochromes with similar spectroscopic properties. During the initial stages of purification it is often difficult to keep track of the cytochrome of interest and we have found that haem staining after SDS electrophoresis to be a valuable method for assessment of the cytochrome composition of groups of chromatographic fractions.

A chromatographic separation of the periplasmic cytochromes of *Ps. stutzeri* is shown to illustrate this point (Fig. 6). The periplasmic cytochromes were adsorbed to DEAE cellulose and eluted with a salt gradient. Equal portions of individual fractions were subjected to SDS electrophoresis treatment followed by haem staining (Fig. 6). Using the profile of A_{410} alone, the presence of overlapping peaks would make the choice of fractions to pool for further purification very difficult. With the aid of the gel, however, all the components of the periplasmic extract (outside lanes) can be detected in the chromatographic profile and fractions containing a particular cytochrome could be combined. For example, the minor component, cytochrome c_4, appears only as a slight shoulder in the chromatographic profile (indicated

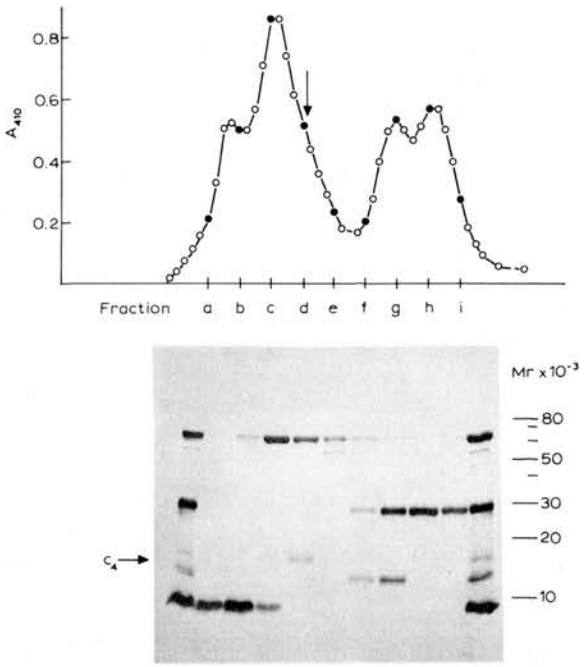

Fig. 6. Ion-exchange chromatography of the periplasmic fraction of denitrifying *Pseudomonas stutzeri*. Equal portions of every fourth fraction (designated a–i) eluted from a DEAE cellulose column were subjected to SDS gel electrophoresis as described in Fig. 3 and haem staining. The cytochrome *c* components of each fraction were compared with the total cytochrome *c* composition of the periplasmic fraction (outer lanes). The arrow indicates a shoulder on the trailing edge of the main chromatographic peak which is shown on the gel to be due to the presence of a small amount of cytochrome c_4. The scale of Mr was constructed from the relative mobilities of a set of molecular-weight marker proteins (not shown).

by an arrow) but, by reference to the gel, its position in the profile can be clearly defined.

Acknowledgements

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Free and membrane-bound forms of bacterial cytochrome c_4

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Cytochrome c_4 was isolated from cells of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Azotobacter vinelandii*. The dihaem nature, M_r of approx. 20000 and ferrohaem spectra in the region of the α- and β-peaks define this family of cytochromes *c*. The behaviour of the holocytochromes in SDS was atypical, but removal of the haem groups resulted in a normal migration. In all three organisms most of the cytochrome c_4 was tightly bound to the membrane, but some free cytochrome was detected. The membrane-attached cytochrome could be extracted with butanol, and this solubilized form was then indistinguishable in properties from the free form. Denitrifying rather than aerobic growth conditions hardly affected the total cytochrome c_4 in the two pseudomonads, but there was slightly more free form and less membrane-attached form in denitrifying growth. The nature of the attachment of cytochrome c_4 to the membrane is discussed and a model is proposed for the process of solubilization.

INTRODUCTION

Many soluble bacterial cytochromes *c* have been characterized (Meyer & Kamen, 1982). It is proposed that these are located in the periplasmic space of Gram-negative bacteria (Wood, 1983), and function as the immediate electron donors to terminal oxidation systems, which may be either free or membrane-attached (Pettigrew & Moore, 1987). However, little attention has been paid to *c*-type cytochromes attached to the membrane. As has been shown in some systems (Ward *et al.*, 1983; Bosma *et al.*, 1987) and as will be apparent in the present paper, there is often a multiplicity of such cytochromes, the functions of which are not known.

Cytochrome c_4 was isolated after butanol extraction from *Azotobacter vinelandii* (Tissières, 1956; Swank & Burris, 1969) and has been reported in some pseudomonads (Ambler & Wynn, 1973; Ambler & Murray, 1973). It is a dihaem cytochrome *c* of M_r approx. 20000, and the amino acid sequences of the cytochromes c_4 from *A. vinelandii* and *Pseudomonas aeruginosa* are known (Ambler, 1980; Ambler *et al.*, 1984). However, in many respects the family is poorly characterized. Thus diagnostic properties have not been identified, the nature of the membrane attachment has not been studied and the association of the cytochrome with different growth conditions is not known. The present paper identifies the characteristic features of the group by studying cytochromes c_4 from four bacteria, namely *A. vinelandii*, *Ps. aeruginosa*, *Pseudomonas stutzeri* and *Alcaligenes* sp. We also investigate the presence and properties of both free and membrane-attached forms of the protein under different growth conditions as a preliminary to a functional characterization of this widespread component of bacterial respiratory chains.

MATERIALS AND METHODS

Growth of bacteria

The organisms used were *Pseudomonas aeruginosa* (N.C.T.C. 10332), *Pseudomonas stutzeri* (Stanier 224, A.T.C.C. 17591), *Azotobacter vinelandii* (strain o, A.T.C.C. 12837) and *Alcaligenes* sp. (formerly *Pseudomonas denitrificans*) (N.C.I.B. 11015).

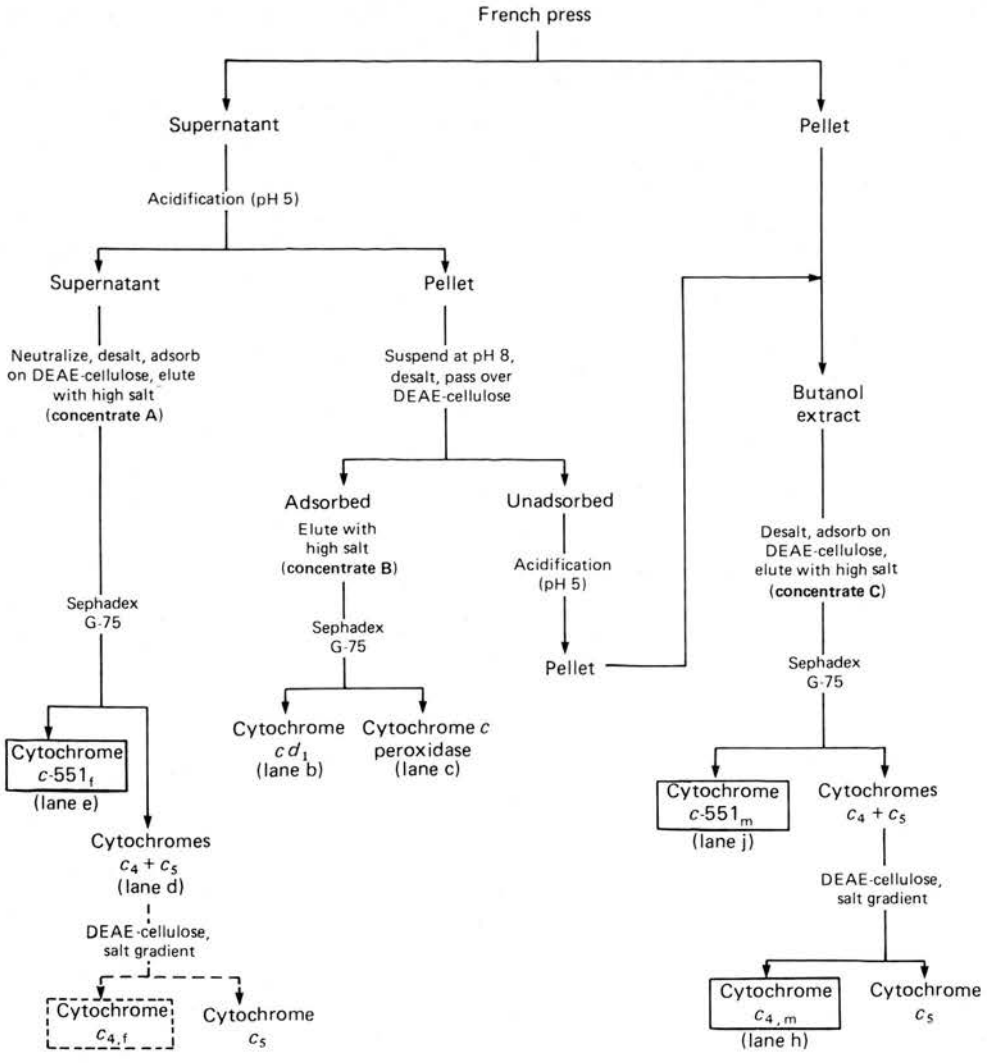
The pseudomonads and *Alcaligenes* sp. were grown aerobically in a 10-litre microfermenter (New Brunswick) in a medium containing trisodium citrate (5 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l) and yeast extract (4 g/l) adjusted to pH 7 at 32 °C. For anaerobic growth with nitrate, cultures were initially grown aerobically in the above medium to a cell density approx. 30% that of the stationary phase, and then $NaNO_3$ (5 g/l) was added aseptically and aeration was stopped. This protocol was found to be essential for denitrifying growth of *Ps. stutzeri*, which did not survive low-level inoculation into nitrate-containing medium (see also the note in the legend to Fig. 8). *A. vinelandii* was grown with vigorous aeration in a modified Burks medium (Newton *et al.*, 1953). Cells were harvested at early stationary phase.

Purification and quantification of cytochromes

The basic strategy for quantification of free and membrane-attached cytochromes *c* is outlined in Scheme 1.

Frozen cells (20–40 g) were thawed, suspended in 5 vol. of 10 mM-sodium phosphate buffer, pH 7, containing 1 mM-$MgCl_2$ and 2 mg of DNAase (type II; Sigma Chemical Co.). The cells were disrupted by passage through a French pressure cell at 83 MPa. In the case of *Ps. aeruginosa*, a second pass through the French press

Abbreviations used: PAc_4, PSc_4, AVc_4 and $ALCc_4$, cytochromes c_4 from *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Azotobacter vinelandii* and *Alcaligenes* sp. respectively.



Scheme 1. Scheme for the separation of cytochromes c_4 and *c*-551 from other cytochromes *c*

Boxes indicate the points at which the amount of a particular cytochrome was determined. Subscripts 'f' and 'm' denote free and membrane-bound species. The lanes indicated in parentheses refer to Fig. 2. The portion of the scheme in broken lines was not carried out with *Ps. aeruginosa* extracts because the amount of cytochrome c_4 was so small.

greatly diminished the viscosity. The broken-cell suspension was centrifuged at 100000 ***g*** for 60 min and the membrane pellet was homogenized in 10 vol. of 5 mM-Tris/HCl buffer, pH 8, and stored frozen. The French-press supernatant was acidified to pH 5 with 1 M-acetic acid and centrifuged at 10000 ***g*** for 30 min. The acidified supernatant was neutralized with 2 M-NH_3, desalted on Sephadex G-25 into 5 mM-Tris/HCl buffer, pH 8, and adsorbed on a DEAE-cellulose (Whatman DE52) column (2.5 cm × 3 cm) equilibrated in the same buffer. The adsorbed material was eluted with 0.2 M-sodium phosphate buffer, pH 7, to give concentrate A (proteins not attached to the membrane and soluble at pH 5).

The acidified pellet was suspended in 10 vol. of water and reneutralized with 2 M-NH_3. This procedure solubilizes any acid-insoluble proteins such as cytochrome cd_1 and cytochrome *c* peroxidase, and the membrane vesicles remained as a fine suspension. The mixture was desalted on Sephadex G-25 into 5 mM-Tris/HCl buffer, pH 8, and adsorbed on a DEAE-cellulose (Whatman DE52) column (2.5 cm × 3 cm) equilibrated in the same buffer. The small membrane vesicles were not adsorbed on this column, and the unadsorbed material was acidified to pH 5 and the precipitate collected by centrifugation as before. These aggregated membranes were added back to the original French-press pellet. The adsorbed material was eluted with 0.2 M-sodium phosphate buffer, pH 7, to give concentrate B (proteins not attached to the membrane and insoluble at pH 5).

The combined membrane suspension in 5 mM-Tris/HCl buffer, pH 8, was homogenized with an equal volume of butan-1-ol (in a top-drive blender), and the emulsion formed was centrifuged at 10000 ***g*** for 30 min. After centrifugation the tubes contained a denatured protein pellet, a red aqueous lower phase, a lipid interface and a butanol upper layer. The butanol still present in the aqueous phase was removed by passage down Sephadex G-25 in 5 mM-Tris/HCl buffer, pH 8, and the cytochromes were adsorbed and eluted from a DEAE-cellulose (Whatman DE52) column as described above to give concentrate C (membrane proteins solubilized by butanol).

Each of the three concentrates was applied separately to a Sephadex G-75 column (2.5 cm × 85 cm) equilibrated with 20 mM-Tris/HCl buffer, pH 8, containing 100 mM-NaCl and previously calibrated with proteins of known M_r values. Fractions in the region of M_r 20000 contained cytochromes c_4 and c_5 and were combined, diluted with 10 vol. of water, reduced with 1 mM-sodium ascorbate and adsorbed on a DEAE-cellulose (Whatman DE52) column (1.5 cm × 10 cm) equilibrated in 5 mM-Tris/HCl buffer, pH 8, containing 1 mM-ascorbate. The adsorbed cytochromes were eluted with a linear salt gradient (400 ml) of 0–100 mM-NaCl in the same buffer. This column separated cytochrome c_4 and cytochrome c_5.

Fractions from the Sephadex G-75 column in the region of M_r 10000 contained cytochrome *c*-551 and no other haem *c* protein.

This standard procedure was applicable to all the extracts studied, with only minor modifications. The quantities of cytochrome *c*-551 and cytochrome c_4 were determined spectrophotometrically after the Sephadex G-75 column stage and the DE52 DEAE-cellulose chromatography respectively.

The strategy of purification and the determination of quantity is summarized in Scheme 1, and, to illustrate the method, profiles of absorbance at 410 nm for the elution pattern from the Sephadex G-75 column for nitrate-grown *Ps. aeruginosa* are shown in Fig. 1. An assessment of the cytochromes *c* present at particular stages was made by haem staining after SDS/polyacrylamide-gel electrophoresis (Fig. 2). In this case cytochrome cd_1 (nitrite reductase) and cytochrome *c* peroxidase were major components of the acid-insoluble proteins of concentrate B (Fig. 1*b* and lanes b and c respectively of Fig. 2). In this organism very little cytochrome c_4 was present unattached to the membrane (Figs. 1*a* and 1*b* and lane d of Fig. 2), and for this reason no DE52 DEAE-cellulose chromatography was carried out. To obtain an estimate of the amount of unattached cytochrome c_4 present, the amount of cytochrome c_4 plus cytochrome c_5 was estimated from the combined fractions in the 20000-M_r region of the Sephadex G-75 column (Fig. 1*a*) and the proportion of cytochrome c_4 was calculated from the relative haem staining of cytochromes

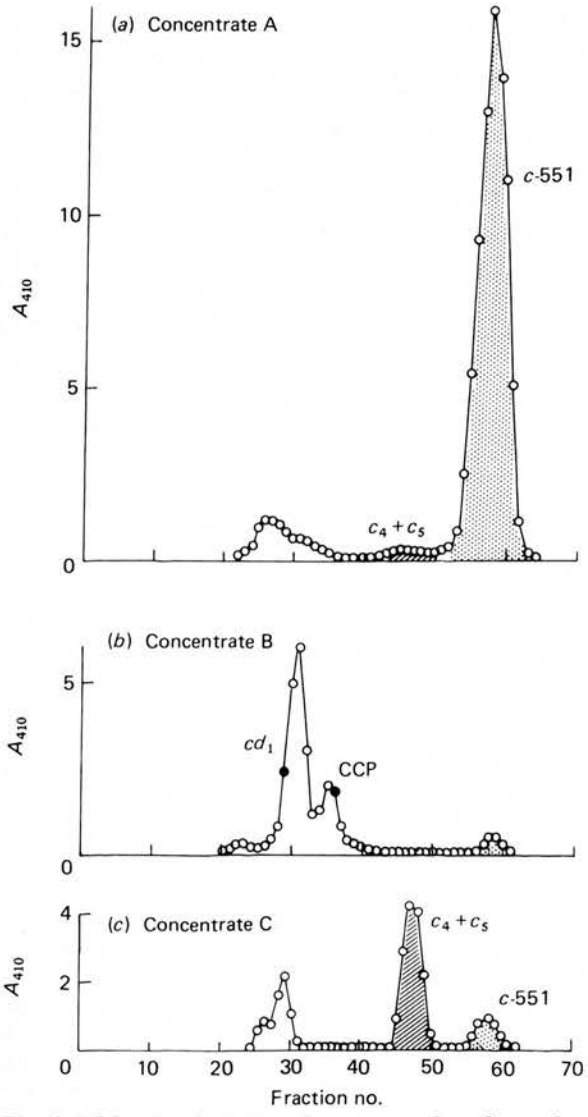


Fig. 1. Molecular-exclusion chromatography of cytochromes *c* from nitrate-grown *Ps. aeruginosa*

The three concentrates described in the Materials and methods section were applied separately to a Sephadex G-75 column (2.2 cm × 85 cm) equilibrated in 20 mM-Tris/HCl buffer, pH 8, containing 100 mM-NaCl, and 3.7 ml fractions were collected. Concentrate A contained cytochromes *c* that were not attached to the membrane and were soluble at pH 5. Concentrate B contained cytochromes *c* that were not attached to the membrane and were insoluble at pH 5. Concentrate C contains cytochromes *c* that were solubilized by butanol from the membrane. The ● symbols denote fractions containing cytochrome cd_1 (panel *b*, fraction 29) and cytochrome *c* peroxidase (CCP) (panel *b*, fraction 36), which were used for the SDS/polyacrylamide-gel electrophoresis of Fig. 2 (lanes b and c respectively).

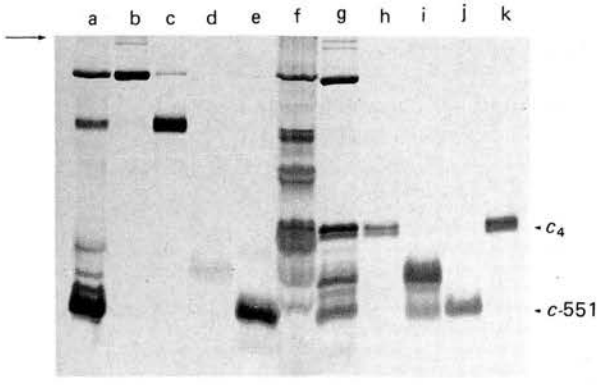


Fig. 2. SDS/polyacrylamide-gel electrophoresis of the cytochromes *c* from nitrate-grown *Ps. aeruginosa*

The arrow indicates the top of the gel (15% acrylamide, 0.4% methylenebisacrylamide). Lane a, concentrate A; lane b, fraction 29 from Fig. 1(*b*); lane c, fraction 36 from Fig. 1(*b*); lane d, pooled cytochrome $c_4 + c_5$ fractions from Fig. 1(*a*); lane e, pooled cytochrome *c*-551 fractions from Fig. 1(*a*); lane f, membranes; lane g, butanol extract; lane h and lane i, from the second and first peaks respectively from the DEAE-cellulose salt-gradient chromatography of the cytochrome $c_4 + c_5$ pooled fractions of Fig. 1(*c*); lane j, pooled cytochrome *c*-551 fractions from Fig. 1(*c*); lane k, purified cytochrome c_4. Bands were detected by haem staining.

c_4 and c_5 after SDS/polyacrylamide-gel electrophoresis (Fig. 2, lane d).

Although the cytochromes c_4 isolated by this procedure were free from other cytochromes, they still contained non-cytochrome proteins. For studies on properties, complete purification of cytochromes c_4 was achieved by using a pH-gradient elution from CM-cellulose (Ambler & Wynn, 1973).

The cytochromes c_4 from *Ps. aeruginosa*, *Ps. stutzeri*, *A. vinelandii* and *Alcaligenes* sp. are below abbreviated to PAc_4, PSc_4, AVc_4 and ALCc_4 respectively.

Haem staining after SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis followed by detection of haem *c* by peroxidase action on 3,5,3′,5′-tetramethylbenzidine was as described by Goodhew *et al.* (1986). This was used as a routine method for the assessment of cytochrome *c* components during purifications, only one example of which is shown (Fig. 2). Protein staining after SDS/polyacrylamide-gel electrophoresis was as described previously (Goodhew *et al.*, 1986).

Spectrophotometry and redox potentiometry

Native spectra were obtained in 0.1 M-sodium phosphate buffer, pH 7, with a Cary 219 spectrophotometer. Pyridine ferrohaemochromes were formed in 2 M-pyridine/0.15 M-NaOH by addition of $Na_2S_2O_4$, and their concentration was determined by using an absorption coefficient at 550 nm of 31.18 $mM^{-1} \cdot cm^{-1}$ (Bartsch, 1971). Potentiometric redox titrations were carried out as previously described (Leitch *et al.*, 1985).

Amino acid analysis

Amino acid analysis of the purified cytochromes was carried out on a Locarte analyser after hydrolysis in 6 M-HCl *in vacuo* for 20–70 h at 105 °C and calculated relative to haem content determined by the pyridine ferrohaemochrome method. Cysteine was measured as cysteic acid after removal of the haem group (Ambler & Wynn, 1973), performic acid oxidation (Hirs, 1967) and acid hydrolysis.

Production of antisera and Western blotting

Antibodies were raised in rabbits by intramuscular injection of 100 μg of purified cytochrome c_4 in Freund's complete adjuvant, followed by two booster injections of 50 μg each after 1 week and 2 weeks. Antisera were collected after 3–4 weeks by bleeding of the ear vein. Western blotting from SDS/polyacrylamide electrophoretic gels was carried out as described in the Bio-Rad Transblot cell instruction pamphlet, with Tween 20 (0.1%) and Marvel milk powder (2%) as blocking agents, 1/100–1/500 dilutions of antisera and detection by goat anti-(rabbit IgG) antibody coupled to horseradish peroxidase (Scottish Antibody Production Unit).

RESULTS

Properties of the purified cytochromes c_4

SDS/polyacrylamide-gel electrophoresis. The oxidized holocytochromes c_4 migrated anomalously fast in SDS/polyacrylamide-gel electrophoresis (Fig. 3). This was especially pronounced with PAc_4, where M_r is approx. 17000 from the gel but is 19900 from the amino acid sequence (Ambler, 1980). However, the cytochrome c_4 could be converted into a slower-migrating form by reduction with 2-mercaptoethanol, which probably results in loss of the ferrous iron from the haem groups (Wood, 1981). A slower-migrating form was also obtained by removal of the haem groups. These effects are shown in Fig. 3 for PSc_4, where the oxidized holocytochrome, 2-mercaptoethanol-reduced cytochrome and apocytochrome have M_r values of 17800, 24500 and 21400 respectively. The cytochrome c_4-like protein from *Alcaligenes* sp. (Shidara, 1980) showed much less of an effect with 2-mercaptoethanol treatment (Fig. 3, lanes a and b).

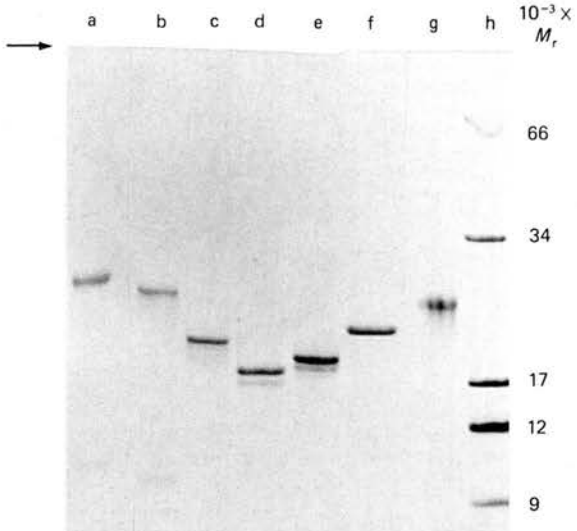


Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified cytochromes c_4

The arrow indicates the top of the gel (15% acrylamide, 0.4% methylenebisacrylamide). Lanes a–f were loaded with 0.35 nmol of purified cytochromes c_4. 2-Mercaptoethanol, when present, was 1% (v/v). Lane a, ALCc_4 + 2-mercaptoethanol; lane b; ALCc_4; lane c, AVc_4; lane d, PAc_4; lane e, PSc_4, lane f, apo-PSc_4; lane g, PSc_4 + 2-mercaptoethanol; lane h, bovine serum albumin (M_r 66000), yeast cytochrome *c* peroxidase (M_r 34000), myoglobin (M_r 17000), cytochrome *c* (M_r 12000) and *Ps. aeruginosa* *c*-551 (M_r 9000). Bands were detected by protein staining.

The holocytochromes c_4 from *Ps. aeruginosa*, *Ps. stutzeri* and *A. vinelandii* were also anomalous in their multiple banding (Fig. 3). This effect was abolished by removal of the haem group (lane f) or by reduction with 2-mercaptoethanol (lane g). In the case of PAc_4, the slower of the two bands could be converted into the faster one by heating the sample at 90 °C in SDS (Fig. 4). This conversion was more complete if the cytochrome was initially in the reduced state (notice that this was pre-reduction followed by removal of the excess reducing agent, unlike the 2-mercaptoethanol treatment described above, which leads to iron loss from the haem in the presence of SDS).

Visible absorption spectra. The α/β regions (500–580 nm) of the ferrocytochrome c_4 spectra are shown in

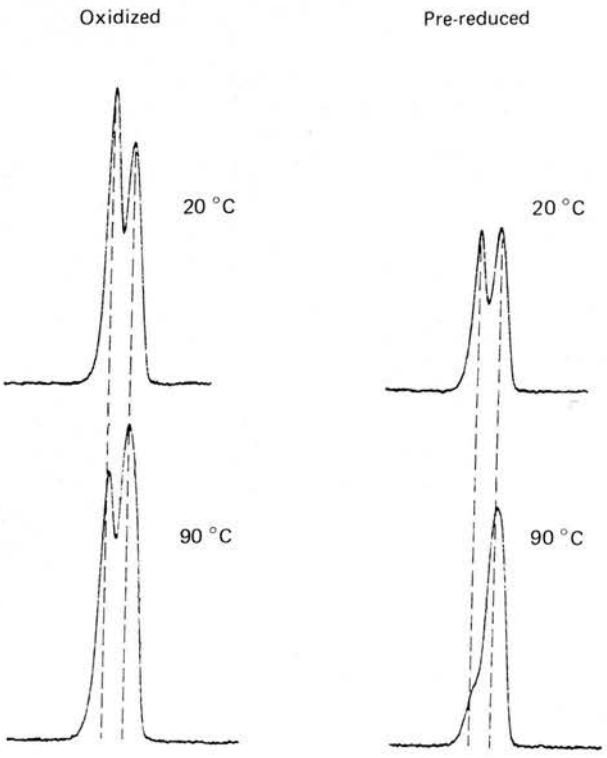


Fig. 4. SDS/polyacrylamide-gel electrophoresis of cytochrome c_4 from *Ps. aeruginosa*

Scans of absorbance at 690 nm in a gel (15% acrylamide, 0.4% methylenebisacrylamide) stained for haem after electrophoresis of samples of *Ps. aeruginosa* cytochrome c_4 were obtained by using a Shimadzu t.l.c. scanner (model CS930). Samples of the oxidized holocytochrome were treated in SDS either at 20 °C or at 90 °C for 4 min. Samples of the reduced holocytochrome, prepared by treatment with 1 mM-ascorbate followed by removal of excess reductant, were similarly treated with SDS either at 20 °C or at 90 °C for 4 min. The top (start) of the gel is to the left of each trace.

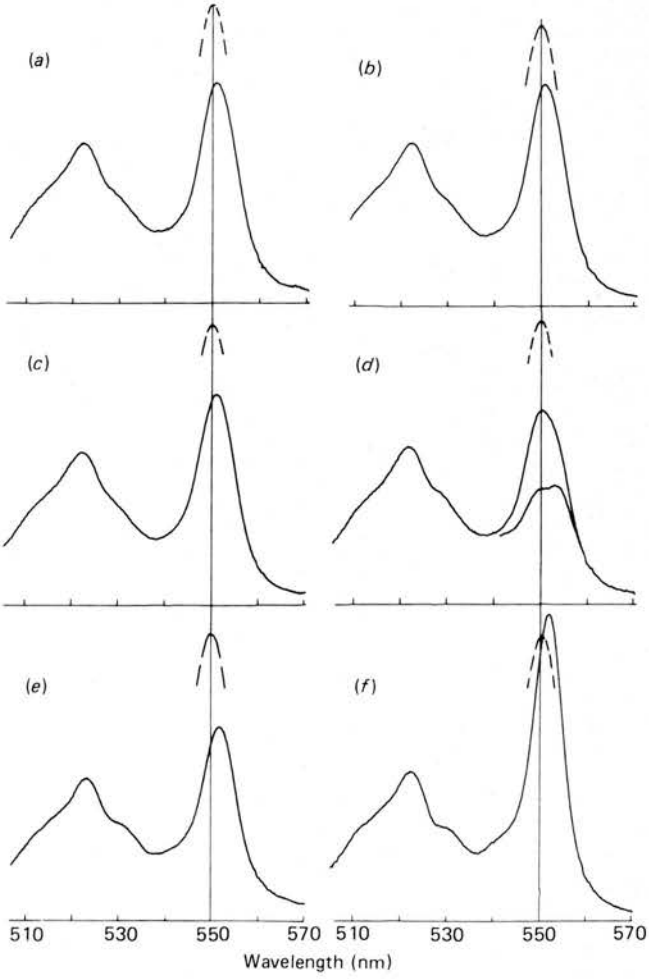


Fig. 5. Visible absorption spectra of the cytochromes c_4 in the region of the α- and β-bands

The native spectra (continuous lines) are for cytochromes c (3.2 μM) reduced with a pinch of $Na_2S_2O_4$ in 0.1 M-sodium phosphate buffer, pH 7. The spectra of pyridine ferrohaemochromes (broken lines) are for cytochromes c at the same concentration reduced with a pinch of $Na_2S_2O_4$ in 2 M-pyridine/0.15 M-NaOH. The peak absorbance of the pyridine ferrohaemochrome spectra is 0.1 (550 nm). (*a*) butanol-solubilized AVc_4; (*b*) free AVc_4; (*c*) butanol-solubilized PAc_4; (*d*) butanol-solubilized PSc_4; (*e*) butanol-solubilized ALCc_4; (*f*) free *Ps. stutzeri* cytochrome *c*-551.

Fig. 5. Included also in the Figure are the pyridine ferrohaemochrome spectra at equivalent concentration (broken lines). The features of low α/β-peak ratio and low α absorbance (see also Table 1) are shared by all the cytochromes c_4 studied, and these features distinguish the group from the cytochromes *c*-551 (e.g. Fig. 5*f*) and most other cytochromes *c*.

Progressive reduction revealed no complexity in the α-peaks of AVc_4 and PAc_4 (Leitch *et al.*, 1985), but with PSc_4 the two haem groups are spectroscopically distinct (Fig. 5*d*). The ferricytochromes c_4 exhibit a near-i.r. band around 700 nm, indicative of methionine–iron co-ordination (Schechter & Saludjian, 1967), and the absorption coefficients (Table 1) suggest that this co-ordination is present for both haem groups.

Amino acid compositions. The amino acid compositions calculated on the basis of haem content by the pyridine ferrohaemochrome method are shown in Table 2. Only a value of 2 mol of haem/mol gave estimates of M_r in agreement with the results of SDS/polyacrylamide-gel electrophoresis.

By using the Cornish-Bowden (1983) approach to the estimation of sequence similarity, PAc_4, PSc_4 and AVc_4 pairings fall below the value of 40% divergence, thus indicating sequence homology (Fig. 6). The c_4-like cytochrome from *Alcaligenes* sp. has a more ambivalent pattern of analysis, although it does show relatedness to AVc_4 (32% predicted sequence divergence).

Immunological cross-reactivity. Western-blot analysis of the purified cytochromes c_4 with antisera raised against AVc_4 and PSc_4 is shown in Fig. 7. Weak cross-reactivity was seen among PAc_4, PSc_4 and AVc_4 (lanes b, c and d) but not against the cytochrome c_4-like protein from *Alcaligenes* sp. (lanes a). This is in agreement with the tenuous sequence similarities predicted in Fig. 6.

Table 1. Spectral and potentiometric properties of the cytochromes c_4

Values in parentheses for the spectral data are millimolar absorption coefficients. Values in parentheses for the potentiometric data are the contributions of each component to the absorbance change at the α-peak. Abbreviation: N.D. not determined.

	AVc_4		PAc_4	PSc_4		$ALCc_4$
	Membrane-bound	Free	Membrane-bound	Membrane-bound	Free	Membrane-bound
α-Peak	550.5 (48.8)	550.5 (48.6)	550.5 (44.2)	550 (44.4)	550.5 (44.2)	551 (45.6)
β-Peak	522 (35.6)	522.5 (35.4)	522 (32.4)	521.5 (36.8)	521.5 (35.9)	522 (39.3)
α/β-Peak ratio	1.37	1.37	1.36	1.21	1.23	1.16
α-Peak/270 nm ratio	1.31	1.29	1.31	1.18	N.D.	1.25
Near-i.r. band	704 (1.7)	N.D.	702 (1.72)	701 (1.7)	N.D.	N.D.
E_m (mV)	317 (56%) 263 (44%)	315 (60%) 240 (40%)	322 (50%) 268 (50%)	300 (41%) 190 (59%)	305 (44%) 215 (56%)	275 (40%) 230 (60%)

Table 2. Amino acid compositions of the cytochromes c_4

The experimental values were obtained as the nmol of amino acids present in a 20 h hydrolysate and calculated relative to the haem content determined by the pyridine ferrohaemochrome method. The sequences for AVc_4 and PAc_4 are from Ambler *et al.* (1984) and Ambler (1980) respectively. The values for cysteine were obtained after oxidation to cysteic acid in the apoprotein. They were calculated relative to phenylalanine. Tryptophan was estimated from the remainder absorbance at 280 nm after subtracting the known contributions of tyrosine residues and the two haem groups. An absorption coefficient of 27.7 $mM^{-1} \cdot cm^{-1}$ (two haem groups) for the latter was derived from spectroscopy of well-characterized cytochromes *c*. Abbreviation: N.D., not determined.

	Amino acid composition (mol/2 mol of haem)					
	AVc_4		PAc_4		PSc_4	$ALCc_4$
Amino acid	Experimental	Sequence	Experimental	Sequence	Experimental	Experimental
Asp	22.9	21	18.4	18	19.3	19.4
Thr	7.9	9	9.7	10	5.5	5.2
Ser	7.8	10	9.6	10	8.6	6.2
Glu	18.1	18	14.8	15	19.8	20.8
Pro	10.6	11	7.1	7	9.2	13.0
Gly	22.2	25	21.3	23	27.8	17.8
Ala	28.7	29	26.9	28	23.0	29.8
Val	5.1	5	5.6	6	7.8	7.6
Met	4.4	6	4	6	4.8	4.8
Ile	5.7	6	7.5	9	4.8	7.4
Leu	12.2	14	13.6	14	14.6	13.4
Tyr	5.8	6	3.7	4	5.2	4.8
Phe	3.7	4	4.9	5	4.0	3.2
His	4.2	4	6.8	6	4.0	3.0
Lys	9.8	11	10.3	11	10.8	8.0
Arg	6.7	7	4.9	5	6.2	5.8
Cys	3.9	4	3.7	4	3.9	N.D.
Trp	0	0	0	0	(0.6)	N.D.
M_r ...		19652		19900	19900	19300

Cytochrome c_4 contents in different cells and under different growth conditions

Cytochromes c_4 from a variety of sources were quantified after a purification scheme designed to minimize the number of steps and therefore optimize the yield. In both pseudomonads under both aerobic and denitrifying conditions, and in *A. vinelandii*, cytochrome c_4 was predominantly membrane-bound, in contrast with the almost entirely unattached cytochrome *c*-551 (Fig. 8). The change from aerobic to denitrifying conditions in both pseudomonads was associated with a lower content of membrane-bound cytochrome c_4 and, in the case of *Ps. stutzeri*, a corresponding increase in the unattached cytochrome c_4 was noted. The relationship between the

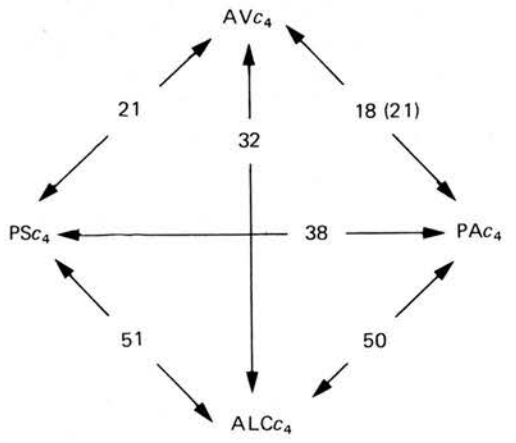


Fig. 6. Prediction of the degree of sequence identity between the cytochromes c_4

The amino acid compositions of the cytochromes c_4 were compared by using the statistical treatment of Cornish-Bowden (1983). Values indicate predicted percentage sequence divergence in paired comparisons. Values below 40% indicate a high probability of sequence relationship (Cornish-Bowden, 1983). The value of 21 in parentheses for the AVc_4–PAc_4 comparison is the actual percentage sequence divergence calculated from the known amino acid sequences (Ambler, 1980; Ambler *et al.*, 1984).

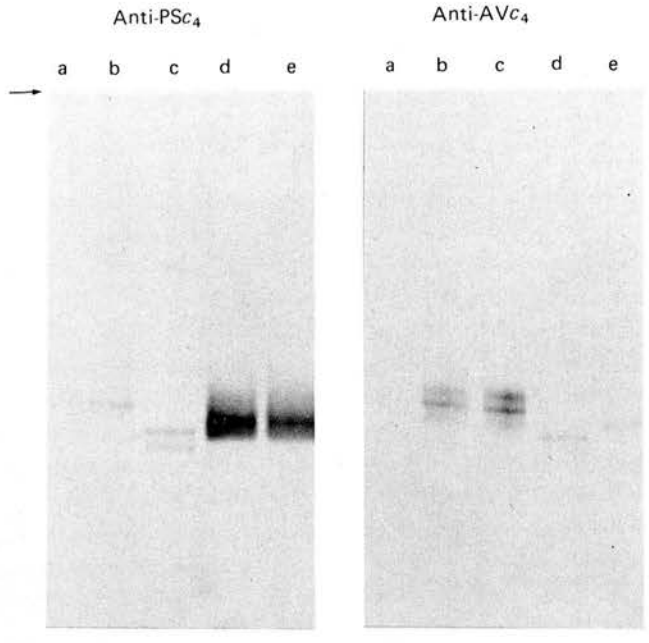


Fig. 7. Western-blot analysis of cytochromes c_4

Oxidized holocytochromes c_4 (25 pmol) after SDS/polyacrylamide-gel electrophoresis (15% acrylamide, 0.4% methylenebisacrylamide) were transferred to nitrocellulose and probed by using antisera raised either against PSc_4 (at 1/500 dilution) or against AVc_4 (at 1/100 dilution). For the former case, the lanes contained: lane a, ALCc_4; lane b, AVc_4; lane c, PAc_4; lane d, butanol-solubilized PSc_4; lane e, free PSc_4. For the anti-AVc_4 experiment the lanes contained: lane a, ALCc_4; lane b, butanol-solubilized AVc_4; lane c, free AVc_4; lane d, PAc_4; lane e, PSc_4. Staining was by a goat anti-(rabbit IgG) antibody coupled to peroxidase in the presence of chloronaphthol and H_2O_2.

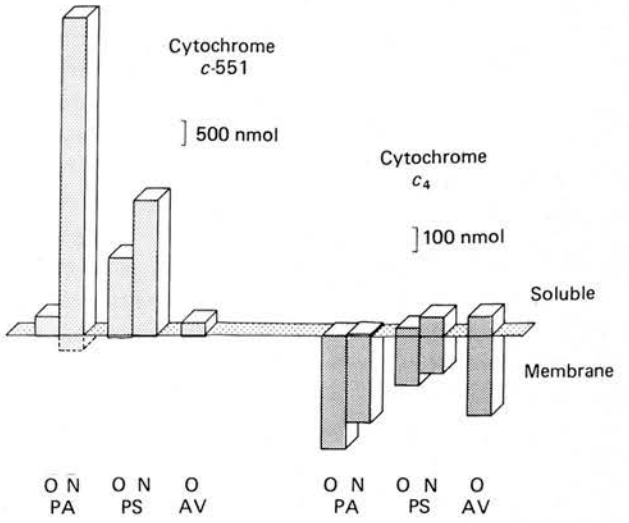


Fig. 8. Contents of cytochromes c_4 and *c*-551 under different growth conditions and in different bacteria

The amounts of cytochrome *c*-551 and cytochrome c_4 were determined spectrophotometrically in the final pooled fractions indicated in Scheme 1, and expressed relative to 100 g wet wt. of cells. *Ps. aeruginosa* (PA) and *Ps. stutzeri* (PS) were grown under aerobic (O) and denitrifying (N) conditions. In the latter mode, because of the high inoculum (30%) of aerobic cells, some proteins associated with aerobic growth may persist in the final stationary denitrifying culture. However, one such protein, a cytochrome *c* of M_r 32000, was found to be present in the denitrifying culture at only 13% the amount found in aerobic cells (Goodhew *et al.*, 1986), indicating that the problem is a small one. *A. vinelandii* can be grown only aerobically. Amounts of free cytochromes are indicated by bars above the horizontal plane; membrane-attached cytochromes are shown by bars below this plane. The broken line for cytochrome *c*-551 in nitrate-grown *Ps. aeruginosa* indicates that the amount of this cytochrome apparently attached to the membrane can be accounted for by free cytochrome in adventitious liquid.

membrane-bound and the unattached cytochromes c_4 is explored further in the following section.

Presence of membrane-bound and free forms of cytochrome c_4

In the cases of *A. vinelandii* and *Ps. stutzeri*, cytochrome c_4 was purified from both the supernatant and pellet of the French-press broken-cell suspensions, indicating the presence of both free and membrane-bound forms. The free cytochrome c_4 in the supernatant was not due to small membrane vesicles, because these could be removed by precipitation at pH 5 and their bound cytochromes *c* were not adsorbed on DEAE-cellulose. However, because of these vesicles, the presence of free cytochrome c_4 cannot be directly demonstrated in French-press supernatants by gel electrophoresis. Free cytochrome c_4 can be directly demonstrated in supernatants prepared by more gentle methods of cell disruption. For example, cytochromes c_4 and *c*-551 were released by treatment of *A. vinelandii* with lysozyme and EDTA (results not shown). The remaining washed membranes still contained the major part of the cytochrome c_4 but almost no cytochrome *c*-551. There was no trace of the distinctive

membrane-bound cytochromes *c* in the supernatants, indicating that these were free of small vesicles. Further washing of the membranes released no more cytochrome, indicating that the free and membrane-bound cytochromes c_4 are not in equilibrium.

In contrast, washing aerobically grown *Ps. aeruginosa* cells by the same method released only trace cytochrome c_4, although most of the cytochrome *c*-551 and cytochrome *c* peroxidase were released (results not shown). This is consistent with the results of the purification studies, which found only very small amounts of free cytochrome c_4 in this organism (Fig. 8).

Comparison of membrane-bound and free forms of cytochrome c_4

The properties of free cytochrome c_4 and cytochrome c_4 released by butanol treatment of membranes were compared for the cases of *Ps. stutzeri* and *A. vinelandii*. Insufficient free cytochrome c_4 could be purified from *Ps. aeruginosa* to allow a comparison.

The free and membrane-bound forms were indistinguishable in their α/β spectra (Figs. 5*a* and 5*b* for AVc_4). Potentiometric redox titrations of cytochromes c_4 reveal the presence of two haem components with different midpoint potentials and different relative contributions to the spectrum (Leitch *et al.*, 1985). Such analyses gave very similar patterns of redox behaviour for the free and butanol-solubilized forms of both PSc_4 and AVc_4 (Table 1).

Free and butanol-solubilized forms of PSc_4 and AVc_4 were indistinguishable by SDS/polyacrylamide-gel electrophoresis with haem or protein staining (results not shown). The two forms also gave similar patterns and intensities of reaction in Western-blot analysis with antisera raised against the butanol-solubilized forms (Fig. 7).

Thus, by the criteria that we have used, the butanol-solubilized and free forms of cytochrome c_4 are indistinguishable in *A. vinelandii* and *Ps. stutzeri*.

DISCUSSION

SDS/polyacrylamide-gel electrophoresis

Cytochromes c_4 behaved anomalously on SDS/polyacrylamide-gel electrophoresis both in their multiple banding patterns and in their relative migration rate. We propose that, even in SDS, the holoprotein retains some compact structure, leading to a faster migration rate in the gel. This compact structure is disrupted by removal of the iron atoms or of the haem groups, allowing complete unfolding of the protein and a lower migration rate on SDS/polyacrylamide-gel electrophoresis. The multiple banding pattern was also abolished by iron or haem removal, and was influenced by heating. This must therefore be a consequence of partially denatured states of the protein rather than heterogeneity in the preparation.

Analysis of cytochrome c_4 contents by purification

Definitive studies on the effect of growth conditions on the contents of particular bacterial cytochromes *c* are rare. Two basic approaches are possible, the first being to examine the whole system without fractionation and the second being to purify the individual components. The first approach includes methods such as gel electrophoresis, redox potentiometry or deconvolution of complex spectra that can suffer from the problem of secure identification. Other proteins may co-migrate on gels or have similar spectra or redox potentials.

The approach that we have used here is that of purification with the strategy of a minimum number of steps to separate cytochrome c_4 from other cytochromes so as to minimize losses. An important feature of our study is the distinction between membrane-bound and unattached forms of cytochromes. We chose a method (French press) that assured complete cell breakage. Although the membrane vesicles produced could in principle contain trapped but unattached cytochromes, we have, in practice, not found this to be a problem with the organisms studied because essentially no cytochrome *c*-551 was found in membrane pellets. Thus all free cytochromes were released into the French-press supernatant.

With such a harsh method of breakage, the supernatant is contaminated with small membrane vesicles. However, this does not lead to an overestimate of unattached cytochrome because that associated with vesicles does not behave in the same way during purification as the protein in the free state. The vesicles do represent losses to the membrane, and this was counteracted by acidification of supernatants and return of the sediments to the membranes.

Although gentler methods such as lysozyme/EDTA washing overcome this problem of small vesicles, we have found it difficult to obtain reproducible quantitative release of soluble cytochromes. Thus in such experiments the membrane pellet is contaminated by soluble but trapped cytochromes *c*.

Occurrence of cytochrome c_4 in different bacteria

From the data presented here, cytochromes c_4 are predominantly membrane-attached proteins with M_r approx. 20000, two high-potential haem groups and a ferrohaem α-peak near 550 nm of low absorptivity. According to these criteria, the 'cytochrome *c*-551' isolated by Shidara (1980) from the *Alcaligenes* species originally called '*Pseudomonas denitrificans*' can be described as a cytochrome c_4. This is in spite of a lack of convincing similarity in amino acid composition (Fig. 6) and an absence of immunological cross-reaction (Fig. 7). Cytochrome c_4 is also present in the strain of *Ps. stutzeri* studied by Kodama & Shidara (1969) and called 'cytochrome *c*-552(II)'. *Pseudomonas perfectomarinus* contains a cytochrome *c*-552 with some of the characteristics of cytochrome c_4 but which is different in spectra and in the very low midpoint potential (−180 mV) of one of the two haem groups (Liu *et al.*, 1981). Clarification of the relationship of this cytochrome to known groups awaits sequence determination, but statistical analysis does predict a low-level sequence divergence (37%) from AVc_4. *Ps. perfectomarinus* also contains a 'cytochrome *c*-551' with low α/β-peak ratio (Liu *et al.*, 1983) that may be a more typical cytochrome c_4. In general, very few studies of bacterial membrane cytochromes *c* have been performed, and cytochrome c_4 may be a more widespread protein than is apparent from its known presence in the pseudomonads, *Alcaligenes* and *A. vinelandii*. For example, Berry & Trumpower (1985) found a membrane-bound *c*-type cytochrome of M_r 22000 in *Paracoccus denitrificans* that requires further characterization.

Nature of the attachment of cytochrome c_4 to the membrane

Most of the cytochrome c_4 in the organisms that we have studied was firmly attached to the membrane and cannot be removed by washing with aqueous salt solutions or chelating agents. Disruption of the lipoprotein membrane by butanol does release cytochrome c_4. This could be by breakage of cytochrome–lipid interactions or of cytochrome–protein interactions. We favour the latter, for the following reasons. Firstly, cytochrome c_4 is not a particularly hydrophobic protein [AVc_4 and PAc_4 have polarity indices (Capaldi & Vanderkooi, 1972) of 42 and 41 % respectively, rather higher than those of most proteins that are integral within the bilayer]. Secondly, significant portions of AVc_4 and PSc_4 were free, which would not be expected for a protein that partitions into the lipid phase. There was no equilibrium partition because no cytochrome c_4 was removed from membranes by further washing.

Thus we propose that cytochrome c_4 is held to the membrane by hydrophobic interactions with a limited number of specific protein sites. Butanol denatures these sites and releases monomeric cytochrome c_4 indistinguishable in properties from cytochrome c_4 that had not been bound to the membrane. Thus the hydrophobic surfaces by which cytochrome c_4 interacts with its membrane protein site may come together in the solubilized two-domain molecule.

Role of cytochrome c_4 in bacterial respiration

In both *Ps. aeruginosa* and *Ps. stutzeri* slightly more cytochrome c_4 was present on the membrane in aerobic compared with nitrate-grown cells (Fig. 8). This, and the fact that cytochrome c_4 is found in the strict aerobe *A. vinelandii*, may indicate that it is involved in aerobic respiration. We propose that a relatively constant amount of cytochrome c_4 is synthesized regardless of growth conditions but that there are more specific sites available for binding to the membrane in the aerobic cells than in the nitrate-grown cells. Once the sites are saturated, cytochrome c_4 appears as a free component. These sites may be the cytochrome *o* of the cytochrome *co*-type oxidase, since Jurtshuk *et al.* (1981) proposed that cytochrome c_4 was the *c*-type component of this enzyme. However, we note that this is a proposal based on the very undiscriminating criterion of membrane spectra, and requires more secure identification of the *c*-type cytochrome.

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